

**Dopaminergic neuroprotective effects of rotigotine via 5-HT1A receptors: possibly
involvement of metallothionein expression in astrocytes**

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1 **Abbreviations used:** ACM, astrocyte conditioned media; ANOVA, analysis of variance;
2 Ara-C, cytosine- β -D-arabinofuranoside; ARE, antioxidant responsive element; DMEM,
3 Dulbecco's Modified Eagle's Medium; DMSO, Dimethyl sulfoxide; ELISA, enzyme-linked
4 immunosorbent assay; FBS, fetal bovine serum; GFAP, glial fibrillary acidic protein; GSH,
5 glutathione; Keap1, Kelch-like ECH-associated protein 1; MT, metallothionein; Nrf2, nuclear
6 factor erythroid 2-related factor; PBS, Phosphate buffered salts; PFA, paraformaldehyde; PD,
7 Parkinson's disease; PMSF, phenyl methylsulfonyl fluoride; RT, room temperature; SD,
8 Sprague-Dawley; SNpc, substantia nigra pars compacta; TH, tyrosine-hydroxylase;
9 WAY100635, N-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-N-(2-pyridinyl)
10 cyclohexanecarboxamide maleate salt; 5-HT_{1A}, serotonin 1A; 6-OHDA, 6-hydroxydopamine

Abstract

Astrocytes exert neuroprotective effects through production of antioxidant molecules and neurotrophic factors. A recent study showed that stimulation of astrocyte serotonin 1A (5-HT1A) receptors promotes astrocyte proliferation and upregulation of the antioxidant molecules metallothionein (MT)-1,2, which protect dopaminergic neurons against oxidative stress. Rotigotine, an anti-parkinsonian drug, can bind to dopamine and 5-HT1A receptors. In this study, we examined neuroprotective effects of rotigotine in models of Parkinson's disease and involvement of astrocyte 5-HT1A receptors in neuroprotective effects of rotigotine against dopaminergic neurodegeneration. Rotigotine increased the number of astrocytes and MT-1,2 expression in cultured astrocytes. Pretreatment with conditioned media from rotigotine-treated astrocytes significantly inhibited 6-hydroxydopamine (6-OHDA)-induced dopaminergic neurotoxicity. These effects were completely blocked by a 5-HT1A antagonist or MT-1,2 specific antibody. Subcutaneous administration of rotigotine increased MT-1,2 expression in striatal astrocytes and prevented reduction of dopaminergic neurons in the substantia nigra of a 6-OHDA-lesioned mouse model of Parkinson's disease. These effects were blocked by co-administration with a 5-HT1A antagonist. These results suggest that rotigotine exerts neuroprotective effects through upregulation of MT expression in astrocytes by targeting 5-HT1A receptors. Our findings provide a possible therapeutic application of rotigotine to prevent dopaminergic neurodegeneration in Parkinson's disease.

1. Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disease. Motor symptoms such as tremor, bradykinesia, and rigidity are induced by degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNpc), which causes depletion of dopaminergic nerve terminals and dopamine (DA) content in the striatum (Chen et al., 2012; Dauer and Przedborski, 2003; Halliday et al., 1996). PD patients also develop non-motor symptoms including psychological manifestations such as depression, and autonomic disorders such as orthostatic hypotension and constipation (Berg et al., 2013; Grinberg et al., 2010; Singaram et al., 1995). Although pathogenesis in sporadic PD has not been characterized, oxidative stress is thought to be an important neurotoxic factor (Wang and Michaelis, 2010). Major medications used to treat PD patients provide dopaminergic therapy for managing motor disability. Therefore, it is essential to develop neuroprotectants that can prevent or delay progression of dopaminergic neurodegeneration.

Astrocytes are abundant neuron-supporting glial cells which comprise blood brain barrier, and regulate cerebral blood flow and synaptic function (Abbott et al., 2006; Araque et al., 1999; Attwell et al., 2010; Gordon et al., 2011; Koehler et al., 2009). In addition, astrocytes exert neuroprotective effects by producing antioxidants and neurotrophic factors, and by taking up excess extracellular glutamate (Banker, 1980; Dringen, 2000; Dringen and Hirrlinger, 2003). Recently, various studies have demonstrated neuroprotective actions of

1 astrocytes (Drukarch et al., 1998; Jewett et al., 2017; Miyazaki and Asanuma, 2016; Nam et
2 al., 2015). We previously reported that striatal astrocytes produced and secreted antioxidant
3 molecules, metallothioneins (MTs), in response to oxidative stress, resulting in protection of
4 dopaminergic neurons (Miyazaki et al., 2011). Stimulation of astrocyte serotonin 1A (5-
5 HT1A) receptors enhances astrocyte proliferation and upregulates MT expression in striatal
6 astrocytes, resulting in protection of dopaminergic neurons in a mouse model of PD
7 (Miyazaki et al., 2013). These findings indicated that astrocyte 5-HT1A receptors may be a
8 novel target for neuroprotection.

9 Rotigotine, a DA agonist, is used to treat PD. Rotigotine binds to all DA receptor
10 subtypes (D1-D5), but has particularly high affinity for D2, D1, and D3 receptors (Wood et
11 al., 2015). In addition to acting as a DA agonist, rotigotine is also a 5-HT1A partial agonist
12 (Scheller et al., 2009). In the present study, we examined neuroprotective effects of rotigotine
13 in a 6-hydroxydopamine (6-OHDA)-lesioned mouse PD model, and explored involvement of
14 astrocyte 5-HT1A receptors in neuroprotective actions of rotigotine.

16 **2. Materials and Methods**

17 **2.1. Animals and animal care**

18 All experimental procedures were conducted following the Guideline for Animal
19 Experiments of Okayama University Advanced Science Research Center, and were approved

by the Animal Care and Use Committee of Okayama University Advanced Science Research Center (approval reference number OKU-2016167). Male Crl:CD1 (ICR) mice and pregnant Sprague-Dawley (SD) rats were purchased from Charles River Japan Inc. (Yokohama, Japan). Mice and rats were housed under temperature- and humidity-controlled condition, which was maintained on a 12 h light/dark cycle with free access to food and water in Okayama University Advanced Science Research Center.

2.2. Cell culture

Primary cultured neurons and astrocytes were prepared from the mesencephalon and striata of SD rat embryos at 15 days of gestation using a previously described method (Miyazaki et al., 2013). To collect astrocyte conditioned media (ACM) and examine effects of rotigotine on MT expression in astrocytes, astrocyte cultures were prepared using striata of rat embryos. Dissected striata were cut into small pieces with scissors, and then incubated in 0.125% trypsin-EDTA (Thermo Fisher Scientific, Waltham, MA, USA) at 37°C for 15 min. After centrifugation (1,500 g for 3 min), the cell pellet was treated with 0.004% DNase I (Sigma-Aldrich, St. Louis, MO, USA) containing 0.003% trypsin inhibitor (Thermo Fisher) at 37°C for 7 min. Following centrifugation (1,500 g for 3 min), cells were plated in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, San Diego, CA, USA) containing 10% fetal bovine serum (FBS) (Sigma-Aldrich) at a density of 2×10^5 cells/cm² on poly-D-lysine-coated

6-well plates (Falcon, Corning, NY, USA). Cells were cultured for 4-7 days in the same medium, then subcultured to obtain enriched astrocyte cultures. Subcultured cells were plated at a density of 3.6×10^4 cells/cm² onto 6-well culture plates (Falcon) for preparation of conditioned media or nuclear protein extraction, or at a density of 2×10^4 cells/cm² onto four-chambered glass culture slides coated with poly-D-lysine (Falcon) for immunohistochemical analysis. After 7 days of culture, greater than 95% cells expressed astrocyte marker proteins.

To prepare enriched neuronal cultures, the mesencephalon was treated with 0.125% trypsin-EDTA and 0.004% DNase I containing 0.003% trypsin inhibitor as described above. Cells were plated in DMEM containing 10% FBS at a density of 2×10^5 cells/cm² on poly-D-lysine-coated four-chambered culture slides (Falcon). The medium was replaced within 24 h with fresh medium supplemented with 2 μ M cytosine- β -D-arabinofuranoside (Ara-C) (Sigma-Aldrich) to inhibit replication of glial cells, and incubated for 6 days. Ninety-five percent of cells were microtubule-associated protein 2-positive neurons, and 1% of the cells were tyrosine hydroxylase (TH)-positive dopaminergic cells.

Mesencephalic neuronal and striatal astrocyte cocultures were prepared by culturing striatal astrocytes for 4 days, then seeding astrocytes at a density of 4×10^4 cells/cm² directly onto mesencephalic neurons that had been cultured on four-chambered culture slides for 4 days. All cultures were maintained at 37°C in a 5% CO₂ atmosphere.

2.3. Preparation of astrocyte conditioned medium (ACM)

Striatal astrocytes were plated onto 6-well plates and grown in DMEM containing 10% FBS for 7 days. Astrocyte conditioned media (ACM) were prepared by treating astrocytes with 1 μ M rotigotine (Sigma-Aldrich) (rotigotine-ACM), 10 nM N-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-N-(2-pyridinyl) cyclohexanecarboxamide maleate salt (WAY100635, a 5-HT_{1A} receptor antagonist, Sigma-Aldrich) (WAY100635-ACM), WAY100635 and rotigotine (WAY100635+rotigotine-ACM), or vehicle (control-ACM) for 24 h. Conditioned media were collected, centrifuged at 3,000 g for 3 min to remove cellular debris, and supernatants were stored at -80 °C until use.

2.4. Cell treatments

Rotigotine was prepared fresh in dimethylsulfoxide (DMSO) (Sigma-Aldrich) for each experiment, then diluted to final concentration in medium (final concentration of DMSO: 0.1% v/v). To investigate the effects of rotigotine on astrocyte proliferation, striatal astrocytes were treated with rotigotine (0.01, 0.1, or 1 μ M) one day after subculture for 24 h, and then counterstained with Hoechst 33342 nuclear stain. To examine effects of rotigotine on MT-1,2 expression in astrocytes, astrocytes cultured for 7 days were treated with rotigotine (0.01, 0.1, or 1 μ M) for 24 h. To study the involvement of the 5-HT_{1A} receptor in rotigotine-induced MT-1,2 up-regulation and Nrf2 nuclear translocation, astrocytes were treated with

1 rotigotine (1 μ M) dissolved in phosphate buffered saline (PBS) with or without WAY100635
2 (10 nM) for 24 h or 6 h respectively. Enriched neuronal cultures and neuron-astrocyte
3 cocultures were treated with rotigotine (1 μ M) for 24 h. After culture media were changed to
4 fresh media, these cells were exposed to 10-150 μ M 6-OHDA (Sigma-Aldrich) for 24 h. To
5 examine whether neuroprotection by rotigotine against 6-OHDA-induced neurotoxicity was
6 mediated by astrocytes, enriched neuronal cultures were treated with control- or rotigotine-
7 ACM for 24 h followed by 6-OHDA exposure. Involvement of astrocyte 5-HT_{1A} receptors in
8 neuroprotection by rotigotine was confirmed by treatment with WAY100635+rotigotine-
9 ACM. For neutralization assay, an anti-MT-1,2 antibody (DAKO Cytomation, Glostrup,
10 Denmark) was preincubated with rotigotine-ACM (1:250; 200 μ g/ml) for 1 h at room
11 temperature (RT), then applied to the neuronal cultures. After 24 h treatment with ACM, or
12 rotigotine-ACM with or without the antibody, culture media were exchanged for fresh media,
13 and neurons were exposed to 6-OHDA (25 μ M) for 24 h. All astrocyte conditioned media
14 contained 2 μ M Ara-C throughout the neuronal culture treatment period.

16 **2.5. Treatment of mice with rotigotine**

17 Healthy male ICR mice weighing 38-42 g (8-week-old) were injected subcutaneously
18 with rotigotine (0.125, 0.25, or 0.5 mg/kg) dissolved in vehicle 2% DMSO in corn oil
19 (NACALAI TESQUE, INC., Kyoto, Japan) once per day for 7 days. All animals were

1 arbitrarily allocated to groups by the experimenter. One day after the final injection of
2 rotigotine, mice were perfused transcardially with 4% paraformaldehyde (PFA) (NACALAI
3 TESQUE) for immunohistochemical analysis under deep pentobarbital anesthesia (80 mg/kg,
4 i.p.) .

6 **2.6. 6-OHDA-lesioned parkinsonian mice and drug treatment**

7 Male ICR mice weighing 38-42 g (8-week-old) were anesthetized by inhalation of
8 isoflurane (Pfizer, Tokyo, Japan) and placed in a stereotaxic apparatus (Narishige, Tokyo,
9 Japan). All mice received unilateral intrastriatal injections of 6-OHDA (7 µg/site, dissolved in
10 1 µl of 0.9% saline containing 0.1% ascorbic acid; Sigma-Aldrich) into three sites in the right
11 striatum at the following coordinates: A +1.2 mm, L +2.0 mm, V +3.0 mm; A +0.9 mm, L
12 +1.4 mm, V +3.0 mm; A +0.5 mm, L +2.0 mm, V +3.0 mm from the bregma, according to a
13 mouse brain atlas (Franklin and Paxinos, 1997). The injection rate was 1 µl/2 min. After
14 injection, the syringe was left in the striatum for an additional 3 min before being retracted
15 slowly. Two weeks after 6-OHDA injections, mice that exhibited asymmetric rotation
16 behavior towards the contralateral side (>30 turns/min) after 0.5 mg/kg apomorphine injection
17 (Sigma-Aldrich) were determined to have developed hemi-parkinsonism. One week after the
18 apomorphine test (3 weeks after 6-OHDA injection), all parkinsonian animals were arbitrarily
19 allocated to groups by the experimenter. Parkinsonian mice were subcutaneously injected

with rotigotine (0.125, 0.25 or 0.5 mg/kg/day) or vehicle 2% DMSO in corn oil for 7 days. To examine whether agonism of 5-HT_{1A} by rotigotine was involved in neuroprotective effects, parkinsonian mice were intraperitoneally injected with the 5-HT_{1A} antagonist WAY100635 (0.25, 0.5 mg/kg/day, Sigma-Aldrich) dissolved in saline 1 h prior to rotigotine treatment. The following exclusion criteria were applied: more than 10% severe weight loss, severe behavioral deficits (paralysis, convulsions), or infections. No animal met the exclusion criteria during the study. One day after final injections, mice were perfused transcardially with 4% PFA for immunohistochemical analysis under deep pentobarbital anesthesia (80 mg/kg, i.p.). For Western blot analysis and ELISA, mice were perfused with ice-cold saline under deep anesthesia, and ventral midbrain and striatum tissues were dissected out immediately.

2.7. Immunohistochemistry

Cells on chamber slides were fixed with 4% PFA for 20 min at RT, blocked with 2.5% normal goat serum for 20 min, and reacted with the following primary antibodies for 18 h at 4°C: mouse anti-TH (1:1,000; Millipore, Temecula, CA, USA), rabbit anti-glial fibrillary acidic protein (GFAP) (1:500; Novus Biologicals, Littleton, CO, USA), or mouse anti-MT-1/-2 antibodies (1:50; Dako Cytomation) diluted in 10 mM PBS containing 0.1% Triton X-100 (WAKO) (0.1% PBST). The secondary antibodies used were goat anti-mouse IgG conjugated to Alexa Fluor 594 (Invitrogen) or goat anti-rabbit IgG conjugated to Alexa Fluor 488

(Invitrogen). Cells were then counterstained with Hoechst 33342 nuclear stain.

For preparation of brain slices, mice were perfused with ice-cold saline followed by a fixative containing 4% PFA under deep pentobarbital anesthesia (80 mg/kg, i.p.). Perfused brains were removed and post-fixed for 24 h in 4% PFA. Following cryoprotection in 15% sucrose in PB for 48 h, brains were snap-frozen with powdered dry ice and 20- μ m-thick coronal sections were cut on a cryostat at levels containing the mid-striatum (+0.6 to +1.0 mm from the bregma) and the SNpc (-2.8 to -3.0 mm from the bregma). Striatal and SNpc sections were incubated in 1% normal goat serum for 30 min at RT, then reacted with mouse anti-MT-1,2 (1:100, DAKO), rabbit anti-MT-1,2 (1:500; Frontier Institute, Hokkaido, Japan) rabbit anti-GFAP (1:20,000, Novus Biologicals), mouse anti-GFAP (1:10,000; Millipore), rabbit anti-S100 β (1:5,000; Dako Cytomation) or mouse anti-S100 β (1:10,000; Sigma Aldrich) antibodies diluted in 0.2% PBST for 18 h at 4°C. After washing in 0.2% PBST, slices were reacted with Alexa Fluor 594- or Alexa Fluor 488-conjugated secondary antibodies, as appropriate. All slides were analyzed using a fluorescence microscope (Olympus, Tokyo, Japan) and a cellSens software imaging system (Olympus) using a mercury lamp through a 470-495 nm, 530-550 nm, or 360-370 nm band-pass filter to excite Alexa Fluor 488, Alexa Fluor 594, or Hoechst dye, respectively. Light emitted from Alexa Fluor 488, Alexa Fluor 594, or Hoechst was collected through a 510-550 nm band-pass filter, a 590 nm long-pass filter, or a 420 nm long-pass filter, respectively. Confocal laser microscope (ZEISS, Oberkochen,

Germany) and ZEN software imaging system (ZEISS) were used to confirm the

colocalization of MT-1,2 and GFAP- or S100 β -positive astrocytes.

For immunostaining of TH in the SNpc, brain slices were treated with 0.5% H₂O₂ (Wako Pure Chemical Corporation) for 30 min at RT, blocked with 1% normal goat serum for 30 min, and incubated with anti-TH rabbit polyclonal antibody (1:1,000, Millipore) diluted in 0.2% PBST for 18 h at 4°C. Slices were incubated with biotinylated goat anti-rabbit IgG (1:1,000; Vector Laboratories, Burlingame, CA, USA) for 2 h followed by avidin-biotin peroxidase complex (1:2,000; Vector Laboratories) for 1 h at RT. TH-immunopositive signals were visualized by 3,3'-diaminobenzidine tetrahydrochloride (Sigma-Aldrich), nickel (Wako Pure Chemical Corporation, Osaka, Japan), and H₂O₂.

For analysis of proliferative activity induced by rotigotine, striatal astrocyte cultures were counterstained with Hoechst 33342 nuclear stain and counted cell number in 8 fields/well chosen arbitrarily under $\times 400$ magnification. Numbers of MT- and GFAP-immunopositive cells in cell cultures were counted in 8-18 fields/well chosen arbitrarily under $\times 200$ magnification, and expressed as the percentages of MT-immunopositive astrocytes to total cell number. The integrated density of MT was calculated as follows: integrated density (signal density – background density) \times area of positive signal. TH-immunopositive cells in cultured cells were counted under a microscope in all areas of each chamber slide.

Cells immunopositive for MT-1,2, GFAP, or S100 β in the dorsal striatum of normal

ICR mice or parkinsonian mice were counted manually using a microscope at a magnification of $\times 200$. The number of MT-1,2-, GFAP-, or S100 β -positive cells and the ratio of MT-1,2-positive cells to GFAP- or S100 β -positive cells were evaluated in each section. The number of TH-immunopositive neurons in the SNpc was counted manually under a microscope at $\times 100$ magnification. The boundary between the SNpc and ventral tegmental area was defined by a line extending dorsally from the most medial boundary of the cerebral peduncle. The ratio of lesion area to the intact side was evaluated. No blinding was performed in the counting of immunopositive cell number and measurement of the immunoreactivity. Broken immunostained slices were excluded from the quantitative analyses.

2.8. Western blot analysis

Nuclear lysates from cultured striatal astrocytes were extracted and prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific Inc., Rockford, IL) according to the protocol provided with the kit. Total cell lysates from ventral midbrain of mice extracted with RIPA buffer (1 mM PBS, pH 7.4, 1% Nonident P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS)) containing phenyl methyl sulfonyl fluoride (PMSF). Western blot analysis was performed as described previously (Miyazaki et al., 2013). In brief, proteins were separated on 10% SDS polyacrylamide gels (Bio-Rad, Hercules, CA, USA) and electrophoretically transferred to polyvinylidene difluoride

membranes (Hybond P, GE Healthcare, UK, Buckinghamshire, UK) at 20 mA/membrane for 60 min. The membranes were pre-incubated with blocking buffer (Block-Ace; DS Bio Pharma Medical, Tokyo, Japan), and incubated with rabbit polyclonal anti-nuclear factor erythroid 2-related factor (Nrf2) (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA), goat polyclonal anti-Lamin B (1:200; Santa Cruz), rabbit polyclonal anti-TH (1:1000; Millipore) or mouse monoclonal anti α -tubulin (1:2000; Sigma-Aldrich) antibodies. After washing with 20 mM Tris-buffered saline containing 0.1% tween 20 (Wako, Tokyo, Japan), the blots were incubated with the corresponding horseradish peroxidase-conjugated secondary antibodies. Signals were visualized via chemiluminescence using an ELC Western blotting detection system (GE Healthcare). Images were obtained and quantified using a FUJIFILM Luminescent Image Analyzer LAS-3000 (FUJIFILM, Tokyo, Japan) and Multi Gauge (v 3.0) software. For quantitative analysis, the signal ratio of Nrf2 (relative chemiluminescence unit) to that of constitutively expressed Lamin B or TH to α -tubulin protein was calculated to normalize for loading and transfer artifacts. The nuclear fraction for Western blot analysis with low concentration was excluded prior to electrophoresis.

2.9. Enzyme-linked immunosorbent assay (ELISA)

Levels of MT-1 in cultured astrocytes and striatal tissue of parkinsonian mice were measured by enzyme-linked immunosorbent assay (ELISA) with mouse MT-1 ELISA kit

(SEB199Mu; Cloud-Clone Corp., Katy, TX, USA) according to the manufacturer's protocol.

To extract total cell lysate from cultured astrocytes or striata of parkinsonian mice for ELISA, cells or tissues were homogenized using RIPA buffer containing PMSF. After centrifugation to remove cellular debris, the supernatants were stored at -80°C until analyses. Protein concentration were determined using the Lowry-based Bio-Rad DC protein assay kit (Bio-Rad) with bovine serum albumin as a standard.

2.10. Statistical analysis

Values were expressed as means \pm SEM. Differences between groups were examined for statistical significance using one-way analysis of variance (ANOVA) followed by *post-hoc* Fisher's PLSD test. A *p* value less than 0.05 was considered statistically significant. The sample size in our study was determined based on our previous reports (Miyazaki et al., 2013).

3. Results

3.1. Rotigotine promoted astrocyte proliferation and upregulated MT-1,2 expression

Treatment with rotigotine (0.1 or 1 μ M) significantly increased the number of striatal astrocytes in cultured cells (Fig. 1A). Rotigotine also upregulated MT-1,2 in striatal astrocytes (Fig. 1B-E). We confirmed colocalization of GFAP and MT-1,2 signals using confocal laser microscope (Fig. 1C). The number of MT-1,2-positive astrocytes is shown in Fig. 1D and

MT-1,2-immunoreactivity is shown in Fig. 1E. These results suggested that rotigotine promoted astrocyte proliferation and upregulated astrocytic antioxidative property in astrocyte cultures.

3.2. Rotigotine induced MT-1,2 expression and Nrf2 nuclear translocation via astrocyte 5-HT1A receptors

To examine whether rotigotine upregulated MT-1,2 in astrocytes via 5-HT1A receptors, striatal astrocyte cultures were treated with rotigotine (1 μ M) and/or the 5-HT1A antagonist WAY100635 (10 nM) for 24 h. WAY100635 can bind to the 5-HT1A receptor with high affinity and selectivity. It is reported that the binding activity of WAY100635 is greater than 100-fold selectivity relative to binding at other 5-HT receptor subtypes and major neurotransmitter receptor, reuptake and ion channel sites (Forster et al., 1995). Simultaneous treatment with WAY100635 almost completely blocked rotigotine-induced MT-1,2 expression as shown in the results of MT-1,2- and GFAP-double immunostaining (Fig. 2A-C). In this study, we measured MT-1,2 levels using total cell lysates from cultured astrocytes by ELISA. Rotigotine significantly upregulated MT levels in astrocytes, but the MT upregulation was not significantly inhibited by WAY100635. (Fig. 2D). MT-1,2 gene expression is regulated by the transcriptional factor Nrf2 (Shih et al., 2003). In the present study, we examined Nrf2 levels in nuclei of astrocytes after 6 h treatment with rotigotine (1 μ M) and

1 WAY100635 (10 nM) by Westernblot analysis. Nrf2 levels were significantly increased by
2 rotigotine treatment, and nuclear translocation of Nrf2 was completely blocked by
3 WAY100635 (Fig. 2E). These results suggested that rotigotine-induced Nrf2 nuclear
4 translocation and MT upregulation occurred through astrocyte 5-HT1A receptor signaling.

6 **3.3. Rotigotine required striatal astrocytes to protect dopaminergic neurons**

7 To examine the role of astrocytes on neuroprotective effects of rotigotine against
8 dopaminergic neurodegeneration, enriched mesencephalic neuronal cultures or mesencephalic
9 neuronal and striatal astrocyte cocultures were treated with rotigotine following by oxidative
10 stress induced by 6-OHDA exposure (Fig. 3A). In enriched neuronal cultures, rotigotine
11 failed to prevent reduction of TH-positive dopaminergic neurons following 6-OHDA
12 exposure (Fig. 3B, C and Suppl. Fig. 1). Conversely, in neuronal and astrocyte cocultures,
13 rotigotine exerted protective effects against 6-OHDA-induced damage of neuritis and
14 reduction of dopaminergic neurons (Fig. 3B, D and Suppl. Fig. 1). These results suggested
15 that neuroprotective effects of rotigotine are mediated by astrocytes.

17 **3.4. Secreted MTs from rotigotine-treated astrocytes via 5-HT1A receptor protected** 18 **dopaminergic neurons against 6-OHDA toxicity**

19 To investigate whether secreted molecules from rotigotine-treated astrocytes can

protect dopaminergic neurons against oxidative stress, enriched neuronal cultures were incubated with conditioned media from striatal astrocytes treated with or without 1 μ M rotigotine (rotigotine-ACM/control-ACM) (Fig. 4A, B). Treatment with rotigotine-ACM significantly inhibited neurite damage and reduction of TH-positive neurons following 6-OHDA exposure. Furthermore, the protective effects of rotigotine-ACM were completely annulled by WAY100635 treatment (Fig. 4D, F and Suppl. Fig. 2), suggesting that rotigotine promoted secretion of neuroprotective molecules from astrocytes via 5-HT_{1A} receptors. MT-1,2 are produced predominantly in astrocytes, and extracellular MTs secreted from astrocytes protect dopaminergic neurons from damage (Miyazaki et al., 2013). Therefore, we examined involvement of MT-1,2 in rotigotine-mediated neuroprotection. Anti-MT-1,2 antibody was added to rotigotine-ACM, and neuronal cultures were treated with rotigotine-ACM containing MT-1,2 antibody followed by 6-OHDA exposure (Fig. 4C). Incubation of rotigotine-ACM with anti-MT-1,2 antibody completely blocked the neuroprotective effects of rotigotine-ACM (Fig. 4E, G and Suppl. Fig. 3). These results suggested that rotigotine exhibits dopaminergic neuroprotection by promoting MT-1,2 secretion from astrocytes via 5-HT_{1A} receptors on astrocytes.

3.5. Rotigotine upregulated MT-1,2 expression in striatal astrocytes in normal mice

To examine whether rotigotine upregulated MT-1,2 expression in astrocytes *in vivo*,

1 healthy ICR mice were subcutaneously injected with rotigotine (0.125, 0.25, 0.5 mg/kg/day)
2 for 7 days (Fig. 5A). Striatal sections were double immunostained with the astrocyte markers
3 S100 β and MT-1,2. MT-1,2 signals were colocalized with S100 β -positive astrocytes (Fig. 5B).
4 The number of MT-1,2-positive astrocytes (Fig. 5C, D) and the ratio of MT-1,2-positive cells
5 to S100 β -positive astrocytes (Fig. 5E) were significantly increased following repeated
6 rotigotine administration.

7 8 **3.6. Rotigotine protected dopaminergic neurons against neurodegeneration in** 9 **parkinsonian mice via 5-HT1A receptor**

10 Rotigotine (0.125, 0.25, 0.5 mg/kg) was administered to 6-OHDA-injected
11 parkinsonian mice to examine neuroprotective effects of rotigotine *in vivo* (Fig. 6A). TH-
12 positive dopaminergic neurons in the SNpc were significantly decreased in the lesioned side
13 of parkinsonian mice. Rotigotine administration (0.5 mg/kg) significantly ameliorated
14 dopaminergic neuronal loss in the SNpc (Fig. 6B, C). To examine whether the 5-HT1A
15 receptor is involved in the neuroprotective effects of rotigotine in parkinsonian mice,
16 rotigotine (0.25 or 0.5 mg/kg/day, s.c.) and/or WAY100635 (0.25 or 0.5 mg/kg/day, i.p.) were
17 administered to 6-OHDA-treated parkinsonian mice for 7 days (Fig. 7A). Rotigotine (0.25,
18 0.5 mg/kg) prevented reduction of TH-positive neurons in the lesioned side of the SNpc.
19 Neuroprotective effects of rotigotine (0.25, 0.5 mg/kg) were blocked by co-administration of

1 WAY100635 (0.5 mg/kg) (Fig. 7B-G). These results suggested that rotigotine protected
2 dopaminergic neurons in parkinsonian mice by targeting 5-HT1A receptors. We also
3 performed TH Western blot analysis using total cell lysate from midbrain tissues containing
4 SNpc of parkinsonian mice after treatment rotigotine (0.25 mg/kg) and/or WAY100635 (0.25,
5 0.5 mg/kg). TH expression in the lesioned side of SNpc was decreased by 30% in contrast to
6 control side. Rotigotine treatment showed neuroprotective tendency, but this did not reach
7 significance (Suppl. Fig.4).

8 9 **3.7. Effects of administration of rotigotine and/or WAY100635 on MT-1,2 expression in** 10 **astrocytes in the striatum of parkinsonian mice**

11 To examine whether rotigotine increased astrocyte MT-1,2 expression in
12 parkinsonian mice, we double immunostained for S100 β and MT-1,2 in striatal sections of
13 parkinsonian mice treated with rotigotine (0.125, 0.25, 0.5 mg/kg). Rotigotine (0.25
14 mg/kg/day, s.c.) administration for 7 days significantly increased the number of MT-1,2-
15 positive astrocytes (Suppl. Fig. 5A, B) and the ratio of MT-1,2-positive cells/S100 β -positive
16 cells in control and lesioned side (Suppl. Fig. 5C). We confirmed colocalization of MT-1,2
17 and S100 β -positive astrocytes in the striatum of parkinsonian mice (Suppl. Fig. 5D). MT
18 induction by rotigotine (0.25 mg/kg/day) treatment in striatal astrocytes was blocked by co-
19 administration of WAY100635 (0.25, 0.5 mg/kg/day, i.p.) (Fig. 8A-D). It is well known that

astrocytes become hypertrophic (i.e., reactive astrocytes) and accumulate in the brain during progression of neurodegenerative diseases. Reactive astrocytes express high levels of GFAP *in vivo*. Therefore, we explored MT-1,2 expression in GFAP-positive reactive astrocytes. Accumulation of GFAP-positive astrocytes was observed in the lesioned side of parkinsonian mice, and MT-1,2 signals were colocalized with GFAP-positive astrocytes (Fig. 9A, B). Rotigotine (0.25 mg/kg/day) treatment significantly increased MT-1,2 expression in reactive astrocytes, and WAY100635 (0.25, 0.5 mg/kg/day) completely blocked this effect (Fig. 9C, D). Repeated rotigotine (0.5 mg/kg/day) administration did not induce MT-1,2 expression in the striatum of parkinsonian mice, and WAY100635 (0.5 mg/kg/day) had no effect on MT expression (Suppl. Fig. 6A-C). Furthermore, we measured MT-1 content by ELISA using striatal total cell lysate from tissues of parkinsonian mice treated with rotigotine (0.25 mg/kg) and/or WAY100635 (0.25, 0.5 mg/kg). There was a tendency to increase in MT content on the lesioned side of parkinsonian mice, but rotigotine failed to induce further increase in MT expression (Suppl. Fig. 7). To examine effects of rotigotine administration on astrocytic MT-1,2 expression in the SNpc, we performed double immunostaining of MT-1,2 and S100 β or GFAP using slices of SNpc of parkinsonian mice administered with rotigotine (0.25 mg/kg) and WAY100635 (0.25 or 0.5 mg/kg). There was no change in MT-1,2 expression in the astrocytes of SNpc in any groups (Suppl. Figs. 8, 9). These results suggested that rotigotine induced MT-1,2 expression in striatal astrocytes, and protected dopaminergic neurons via 5-

HT1A receptor signaling in parkinsonian mice.

4. Discussion

In the present study, we demonstrated dopaminergic neuroprotective effect of rotigotine via 5-HT1A receptor in parkinsonian models. Rotigotine administration prevented dopaminergic neurodegeneration in parkinsonian mice, which were assessed by counting TH-positive cells. However, the results of TH Western blot analysis using tissue samples showed neuroprotective tendency, but there was no significance. By immunohistochemistry, TH-positive signal in each neuron in the lesioned side of 6-OHDA-injected mice was stronger than that in the control side. These observations suggest compensatory increase of TH expression in the lesioned-side SN of parkinsonian mice. Western blot analysis reflects expression of TH protein but not neuronal survival. Therefore, we suppose it is difficult to reproduce the results of TH immunostaining by Western blot analysis.

We showed that upregulation of MT-1,2 in astrocytes could be involved in rotigotine-mediated neuroprotection in this study. MT is a cysteine-rich protein, which has strong antioxidative, anti-apoptotic, and anti-inflammatory properties (Giralt et al., 2002; Murakami et al., 2014; Pan et al., 2013; Penkowa, 2006; Ruttkay-Nedecky et al., 2013). In the brain, two major isoforms, MT-1 and -2, are expressed mainly in astrocytes and secreted in response to oxidative stress (Chung et al., 2004). We previously demonstrated that astrocytes

1 produced MT-1,2 in response to excess DA-induced oxidative stress, and extracellular MT-
2 1,2 protected dopaminergic neurons against DA-induced neurotoxicity (Miyazaki et al., 2011).
3 In addition, MT-1 binds DA quinone, a dopaminergic neuron-specific oxidative stress product
4 formed by DA oxidation, and can protect dopaminergic neurons against DA quinone
5 neurotoxicity (Asanuma et al., 2003; Graham, 1978; Hastings et al., 1996; Miyazaki et al.,
6 2007; Rabinovic and Hastings, 1998). It is well known that 6-OHDA induces dopaminergic
7 neurotoxicity via reactive oxygen species. Previous studies reported that 6-OHDA also
8 produce *p*-quinone by auto-oxidation, played a pivotal role in 6-OHDA-induced neurotoxicity,
9 and glutathione (GSH) conjugated with *p*-quinone and provided neuroprotection (Izumi et al.,
10 2005). Taken together with these previous reports, it is suggested that up-regulation of MT-
11 1,2 expression induced by rotigotine can provide neuroprotection against oxidative stress-
12 induced dopaminergic neurodegeneration. It is also supposed by a previous report, which
13 demonstrated that rotigotine decreased reactive oxygen species in rotenone-treated primary
14 cultured cells from midbrain (Radad et al., 2014). The involvement of up-regulation of anti-
15 oxidative property in neuroprotective effects of rotigotine would be examined in the future
16 study. In this study, we showed that MT expression was increased after
17 rotigotine treatment via 5-HT_{1A} receptor, which were assessed by counting the number of
18 MT-1,2-positive astrocytes. Unlike the results of immunohistochemistry using brain slices,
19 ELISA showed increasing tendency of MT content in the lesioned-side striatum of

parkinsonian mice, and rotigotine (0.25 mg/kg) failed to induce further increase in MT expression. As mentioned above, MT-1,2 are expressed mainly in astrocytes and protein secreted from astrocytes are consumed by neurons to reduce oxidative stress. Therefore, we suppose that it is difficult to detect changes in MT expression using tissue homogenates of parkinsonian mice. As shown in this study, MT-1,2 were induced specifically in GFAP-positive reactive astrocyte. Because astrocytes were activated dramatically in the lesioned-side striatum of 6-OHDA-injected mice, we could observe MT upregulated at least in the lesioned side.

We demonstrated that rotigotine promoted Nrf2 nuclear translocation via astrocyte 5-HT_{1A} receptor, resulting in increased MT-1,2 expression. Nrf2 is a master transcription factor, which responds to oxidative stress and induces various antioxidative molecules including MT-1,2 (Miyazaki et al., 2011; Suzuki et al., 2013a; Suzuki et al., 2013b). GSH is also synthesized in astrocytes regulated by Nrf2 and secreted into the extracellular space to protect neurons against oxidative stress. Previous studies showed that GSH levels are lower in the SN of PD patients compared to those in control subjects (Jenner et al., 1992; Riederer et al., 1989; Sian et al., 1994; Sofic et al., 1992). Therefore, we examined GSH content in conditioned media from roigotine-treated astrocytes. However, GSH levels in rotigotine-ACM were not different from levels in control-ACM (data not shown).

Previously, we reported that treatment with 5-HT_{1A} full agonist promoted astrocyte

proliferation via secretion of S100 β *in vitro* and *in vivo* (Miyazaki et al., 2013). S100 β is a calcium binding protein and expressed primarily in astrocytes. Stimulation of 5-HT1A receptor on astrocytes promotes S100 β secretion, and extracellular S100 β exerts autocrine effects that promote astrocyte proliferation. To assess effect of rotigotine on astrocyte proliferation, we counted astrocytes in cultured cells or normal or parkinsonian mice. In the present study, we used anti-GFAP and anti-S100 β antibodies to detect astrocytes by immunohistochemistry. In cell cultures, all astrocytes express GFAP, so we counted GFAP-positive cells. On the other hands, an anti-GFAP antibody detected mainly fibrous activated astrocytes in the brain of mice. To assess all types of astrocytes, including protoplasmic astrocytes *in vivo*, we used anti-S100 β antibody. Treatment with rotigotine (0.1, 1 μ M) increased the number of GFAP-positive cultured astrocytes. Rotigotine also showed the increasing tendency of S100 β -positive astrocyte number in normal mice and control side of parkinsonian mice. However, the number of GFAP-positive activated astrocytes was not changed by rotigotine. These results suggested that rotigotine has weak proliferation effect of astrocyte, but not astrocyte activation.

In this study, we used two doses of rotigotine (0.25 or 0.5 mg/kg). In contrast to rotigotine (0.25 mg/kg)-treated group, rotigotine (0.5 mg/kg) did not upregulate MT-1,2 expression, but the drug could inhibit the reduction of nigral dopaminergic neurons in parkinsonian mice. These data suggest that rotigotine may activate several neuroprotective

1 pathways including not only Nrf2-MT pathway but also other pathways, which are not
2 regulated by Nrf2, via 5-HT1A receptor. In addition, rotigotine (0.25 mg/kg) induced MT
3 expression rather than rotigotine (0.5 mg/kg). In the previous study, we reported that effects
4 of 5-HT1A full agonist on astrocytes are not exhibited in a dose-dependent manner (Miyazaki
5 et al., 2013). There is an optimal dose of 5-HT1A agonist to activate astrocytic antioxidative
6 property. Taken together, we suppose rotigotine (0.5 mg/kg) may affect other neuroprotective
7 pathways rather than MT upregulation. WAY100635 (0.25 mg/kg) administration could annul
8 rotigotine (0.25 mg/kg)-induced MT upregulation in astrocytes, but not dopaminergic
9 neuroprotective effect. As mentioned above, we demonstrated that rotigotine induced Nrf2
10 translocation to nuclei via 5-HT1A receptor. Taken together with these results from *in vivo*
11 and *in vitro*, it is suggested that rotigotine exerts neuroprotective effects by not only MT
12 upregulation but also other mechanisms induced by 5-HT1A receptor stimulation. We
13 supposed that WAY100635 (0.25 mg/kg) is not sufficient to block all neuroprotective
14 mechanisms.

16 **5. Conclusion**

17 The present study demonstrated that the anti-parkinsonian agent, rotigotine,
18 increased expression of antioxidant MTs in astrocytes and protected dopaminergic neurons
19 against oxidative stress via 5-HT1A receptors. Furthermore, administration of rotigotine

1 prevented dopaminergic neurodegeneration in parkinsonian mice. These results suggested that
2 rotigotine upregulates antioxidant molecules by targeting 5-HT1A receptors on astrocytes,
3 resulting in neuroprotection. Our findings suggested that rotigotine may be effective as a
4 disease-modifying treatment for PD.

6 **Acknowledgements and conflict of interest disclosure**

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15 All experiments were conducted in compliance with the ARRIVE guidelines.

17 **Supporting information**

18 Additional supporting information may be found online in the Supporting
19 Information section at the end of the article.

Suppl. Fig. 1 Representative photomicrographs of TH immunostaining. Mesencephalic neuronal culture or mesencephalic neuron and striatal astrocyte coculture were treated with rotigotine (1 μ M) for 24 h, and then exposed to 6-OHDA for 24 h.

Suppl. Fig. 2 Representative photomicrographs of TH immunostaining. Rotigotine and/or WAY100635 were added to astrocytes cultures and incubated for 24 h. Astrocyte conditioned media (ACM) were collected, and mesencephalic neurons were pre-treated with ACM (control-ACM, rotigotine-ACM, or WAY100635+rotigotine-ACM) for 24 h prior to 6-OHDA exposure.

Suppl. Fig. 3 Representative photomicrographs of TH immunostaining. Rotigotine was added to astrocytes cultures and incubated for 24 h. Astrocyte conditioned media (ACM) were collected, and anti-MT-1,2 antibody was added to rotigotine-ACM to neutralize secreted MT-1,2 from rotigotine-treated astrocytes. Mesencephalic neurons were treated with rotigotine-ACM containing anti-MT-1,2 antibody for 24 h, and then exposed to 6-OHDA.

Suppl. Fig. 4 Effects of administration of rotigotine (0.25 mg/kg, s.c.) and WAY100635 (0.25, 0.5 mg/kg, i.p.) for 7 days on TH expression in the ventral midbrain of parkinsonian mice.

Suppl. Fig. 5 Effects of rotigotine (0.125, 0.25, 0.5 mg/kg/day, s.c.) administrations for 7 days on MT-1,2 expression in the striatal astrocytes in parkinsonian mice.

Suppl. Fig. 6 Effects of administration of rotigotine (0.5 mg/kg/day, s.c.) and WAY100635 (0.5 mg/kg/day, i.p.) for 7 days on MT-1,2 expression in the striatum of parkinsonian mice.

Suppl. Fig. 7 Change in MT-1 content in total cell lysate from striatum of parkinsonian mice after treatment rotigotine (0.25 mg/kg, s.c.) and/or WAY100635 (0.25, 0.5 mg/kg, i.p.).

Suppl. Fig. 8 Effects of administration of rotigotine (0.5 mg/kg/day, s.c.) and WAY100635 (0.5 mg/kg/day, i.p.) for 7 days on MT-1,2 expression S100 β -positive astrocytes in the SNpc of parkinsonian mice.

Suppl. Fig. 9 Effects of administration of rotigotine (0.5 mg/kg/day, s.c.) and WAY100635 (0.5 mg/kg/day, i.p.) for 7 days on MT-1,2 expression in GFAP-positive astrocytes in the SNpc of parkinsonian mice.

Author contributions

I.M., M.A. designed the research; N.I., I.M., R.K., K.W., E.N., K.S. and D.Y. performed experiments and analyzed the data; Y.K. assisted with some of the *in vivo* experiments; N.I., I.M. and M.A. wrote the paper; M.A. organized the project.

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7

Figure legends

Fig. 1 Effects of rotigotine treatment on astrocyte proliferation and MT-1,2 expression.

Cultured striatal astrocytes were treated with rotigotine (0.01, 0.1, 1 μ M) for 24 h. (A)

Number of GFAP-positive astrocytes after rotigotine treatment. Each value is represented as

the mean \pm SEM (n = 3), expressed as a percentage of the control. (B) Representative

photomicrographs of MT-1,2 and GFAP double immunostaining. Scale bar: 50 μ m. (C)

Photomicrographs of confocal laser microscope. Scale bar: 100 μ m. (D, E) Rotigotine

upregulated MT-1,2 expression in astrocytes. (D) Ratio of MT-1,2-immunopositive astrocytes

to total cells. (E) Integrated density of MT-1,2 immunoreactivity. Data are expressed as

means \pm SEM (n = 3). *p < 0.05, **p < 0.01 vs. control group.

Fig. 2 Rotigotine upregulated MT-1,2 expression and promoted nuclear translocation of

Nrf2 in astrocytes via 5-HT1A receptors.

(A-D) Effects of treatment with rotigotine and/or WAY100635 on MT-1,2 expression in

astrocytes. Cultured striatal astrocytes were treated with rotigotine (1 μ M) and/or

WAY100635 (10 nM) for 24 h. (A) Representative photomicrographs of MT-1,2 and GFAP

double immunostaining. Scale bar: 50 μ m. (B) Ratio of MT-1,2-immunopositive astrocytes to

total cells. (C) Integrated density of MT-1,2 immunoreactivity. Data are expressed as means \pm

SEM (n = 3). (D) Changes in MT-1 content in cultured astrocytes after treatment with

rotigotine (1 μ M) and/or WAY100635 (10 nM) for 24h. MT-1 content was determined by ELISA. Data expressed as means \pm SEM (n = 6). (E) Effects of treatment with rotigotine and/or WAY100635 on nuclear Nrf2 expression in astrocytes. Data are means \pm SEM (n = 4-6). *p < 0.05, ***p < 0.001 vs. control group, #p < 0.05, ##p < 0.01, ###p < 0.001 vs. the rotigotine-treated group.

Fig. 3 Rotigotine protected dopaminergic neurons against 6-OHDA toxicity by targeting astrocytes.

(A) Flow chart of the study design. Mesencephalic neuronal culture or mesencephalic neuron and striatal astrocyte coculture were treated with rotigotine (1 μ M) for 24 h, and then exposed to 6-OHDA for 24 h. Cells were fixed and immunostained for TH. (B) Morphological change of dopaminergic neurons 24h after 6-OHDA exposure. Scale bar: 50 μ m. TH-positive cell number in enriched mesencephalic neuronal cultures (C) or mesencephalic neuronal and striatal astrocyte cocultures (D) Each value is presented as the mean \pm SEM (n = 3), expressed as percentage of control. **p < 0.01, ***p < 0.001 vs. each control group, ##p < 0.01, ###p < 0.001 between the two indicated groups.

Fig. 4 Rotigotine promoted MT-1,2 secretion from astrocytes to protect dopaminergic neurons via 5-HT1A receptors.

(A-C) Flow chart of the study design. Rotigotine and/or WAY100635 were added to astrocytes cultures and incubated for 24 h. Astrocyte conditioned media (ACM) were collected, and mesencephalic neurons were pre-treated with ACM (control-ACM, rotigotine-ACM, or WAY100635+rotigotine-ACM) for 24 h prior to 6-OHDA exposure (A, B). Anti-MT-1,2 antibody was added to rotigotine-ACM to neutralize secreted MT-1,2 from rotigotine-treated astrocytes. Mesencephalic neurons were treated with rotigotine-ACM containing anti-MT-1,2 antibody for 24 h, and then exposed to 6-OHDA (A, C). (D, E) Morphological change of dopaminergic neurons 24h after 6-OHDA exposure treated with rotigotine-ACM and/or WAY100635 (D) or rotigotine-ACM+MT-1,2 antibody (E). Scale bar: 50 μ m. (F) Cell viability of TH-positive dopaminergic neurons after treatment with rotigotine-ACM and/or WAY100635. Neuroprotective effects of conditioned media from rotigotine-treated astrocytes (rotigotine-ACM) were blocked by WAY100635 treatment (WAY100635+rotigotine-ACM). (G) Incubation of rotigotine-ACM with anti-MT-1,2 antibody (rotigotine-ACM+MT-1,2 Ab) blocked neuroprotective effects of rotigotine-ACM. Data are presented as means \pm SEM (n = 4). **p < 0.01, ***p < 0.001 vs. each control group, ###p < 0.001 between the two indicated groups.

Fig. 5 Effects of rotigotine treatment on MT-1,2 expression in astrocytes in the striatum of normal mice.

(A) Flow chart of the study design. Rotigotine (0.125, 0.25, 0.5 mg/kg/day) or vehicle (2% DMSO in corn oil) was administered subcutaneously to healthy ICR mice (male, 8 weeks old) for 7 days. Mice were perfused with a fixative one day after the final administration. (B) Images of confocal laser microscope. Scale bar: 100 μ m. (C) Representative photomicrographs of MT-1,2 and S100 β double immunostaining using striatal slices. Scale bar: 100 μ m. (D) Number of S100 β - and MT-1,2-positive cells. (E) Ratio of MT-1,2 immunopositive astrocytes to total cells. Each value is presented as the mean \pm SEM (n = 5). *p < 0.05, **p < 0.01, ***p < 0.001 vs. the vehicle-treated group.

Fig. 6 Neuroprotective effects of rotigotine administration against dopaminergic neurodegeneration in parkinsonian mice.

(A) Flow chart of the study design. 6-OHDA was administered to the right striatum of ICR mice (male, 8 weeks old) to prepare parkinsonian mice. Fourteen days after 6-OHDA injection, apomorphine rotation test was performed to confirm development of parkinsonism. Rotigotine (0.125, 0.25, 0.5 mg/kg/day) or vehicle (2% DMSO in corn oil) was administered subcutaneously to the parkinsonian mice for 7 days. Parkinsonian mice were perfused with a fixative one day after the final administration. (B) Representative photomicrographs of TH immunostaining in the SNpc of parkinsonian mice after repeated administration of rotigotine for 7 days. Scale bar: 200 μ m. (C) Ratio of nigral TH-immunopositive cells in the lesioned

side to the control side of parkinsonian mice after treatment with rotigotine (0.125, 0.25, 0.5 mg/kg/day, s.c.) or vehicle (2% DMSO in corn oil) for 7 days. Each value is presented as the mean \pm SEM (n = 5). *p < 0.05, **p < 0.01 vs. the control side of each group, #p < 0.05 vs. the vehicle-treated group.

Fig. 7 Rotigotine protected dopaminergic neurons via 5-HT_{1A} receptors in parkinsonian mice.

(A) Flow chart of the study design. Parkinsonian mice were administrated with WAY100635 (0.25, 0.5 mg/kg/day, i.p.) or vehicle (saline) 1 h prior to rotigotine treatment (0.25, 0.5 mg/kg/day, s.c.). One day after the final administration, mice were perfused with a fixative.

(B, C, F) Effects of administration of rotigotine (0.25 mg/kg/day, s.c.) and/or WAY100635 (0.25, 0.5 mg/kg/day, i.p.) for 7 days on nigral TH-positive neurons in parkinsonian mice. (B)

Representative photomicrographs of TH immunostaining in the SNpc. Scale bar: 200 μ m. (C)

High magnification images of (B). Scale bar: 100 μ m. (F) Ratio of TH-immunopositive cells

in the lesioned side to the control side. Each value is presented as the mean \pm SEM (n = 6-7

mice per group from one independent experiment). **p < 0.01 vs. the control side of each

group, #p < 0.05 between the two indicated groups. (D, E, G) Effects of administration of

rotigotine (0.5 mg/kg/day, s.c.) and/or WAY100635 (0.5 mg/kg/day, i.p.) for 7 days on nigral

TH-positive neurons in parkinsonian mice. (D) Representative micrographs of TH

immunostaining in the SNpc. Scale bar: 200 μ m. (E) High magnification images of (D). Scale bar: 100 μ m. (G) Ratio of TH-immunopositive cells in the lesion side to the control side. Each value is presented as the mean \pm SEM (n = 5). [#]p < 0.05 between the two indicated groups.

Fig. 8 Rotigotine upregulated MT-1,2 expression in S100 β -positive striatal astrocytes in parkinsonian mice via 5-HT1A receptors.

Effects of administration of rotigotine (0.25 mg/kg/day, s.c.) and/or WAY100635 (0.25, 0.5 mg/kg/day, i.p.) for 7 days on MT-1,2 expression in striatal astrocytes in parkinsonian mice.

We performed double-immunostaining of S100 β and MT-1,2. (A) Images of confocal laser microscope. Scale bar: 100 μ m. (B) Representative photomicrographs of S100 β and MT-1,2 double immunostaining. Scale bar: 50 μ m. (C) Number of S100 β - and MT-1,2-positive cells in the striatum of parkinsonian mice. (D) Ratio of MT-1,2-positive cells to S100 β -positive cells. Data are presented as means \pm SEM (n = 5). *p < 0.05 vs. the same side of the vehicle-treated group, [#]p < 0.05, ^{##}p < 0.01 vs. the same side of the rotigotine-treated group.

Fig. 9 Rotigotine upregulated MT-1,2 expression in GFAP-positive astrocytes in the striatum of parkinsonian mice via 5-HT1A receptors.

Effects of administration of rotigotine (0.25 mg/kg/day, s.c.) and/or WAY100635 (0.25, 0.5 mg/kg/day, i.p.) for 7 days on MT-1,2 expression in striatal astrocytes in parkinsonian mice.

1 We performed double immunostaining of GFAP and MT-1,2. (A) Images of confocal laser
2 microscope. Scale bar: 100 μ m. (B) Representative photomicrographs of GFAP and MT-1,2
3 double immunostaining. Scale bar: 50 μ m. (C) Number of GFAP- and MT-1,2-positive cells
4 in the striatum of parkinsonian mice. (D) Ratio of MT-1,2-positive cells to GFAP-positive
5 cells on the lesioned side. Data are presented as means \pm SEM (n = 5). *p < 0.05 vs. the same
6 side of the vehicle-treated group, #p < 0.05, ##p < 0.01 vs. the same side of the rotigotine-
7 treated group.

Fig. 1

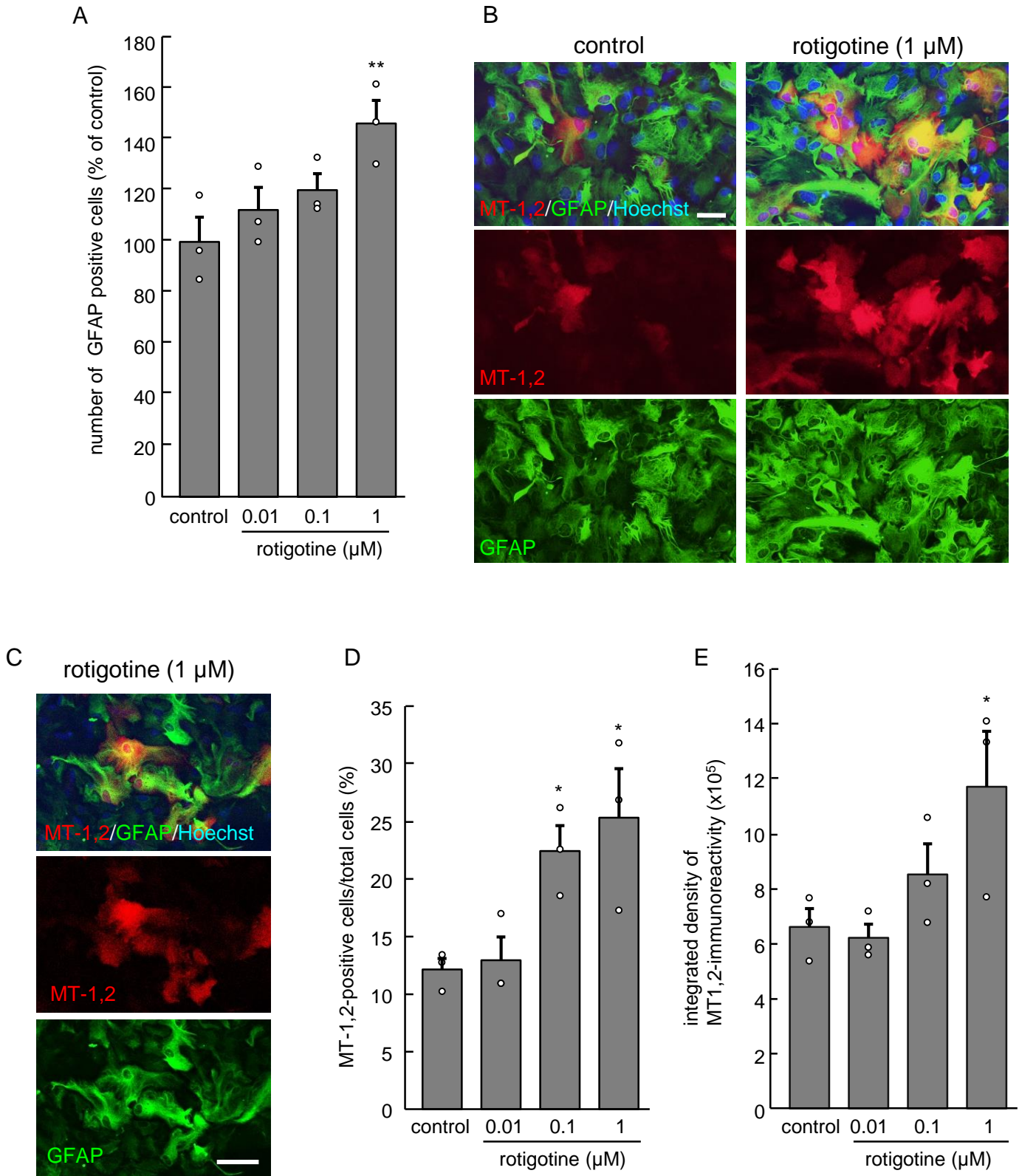


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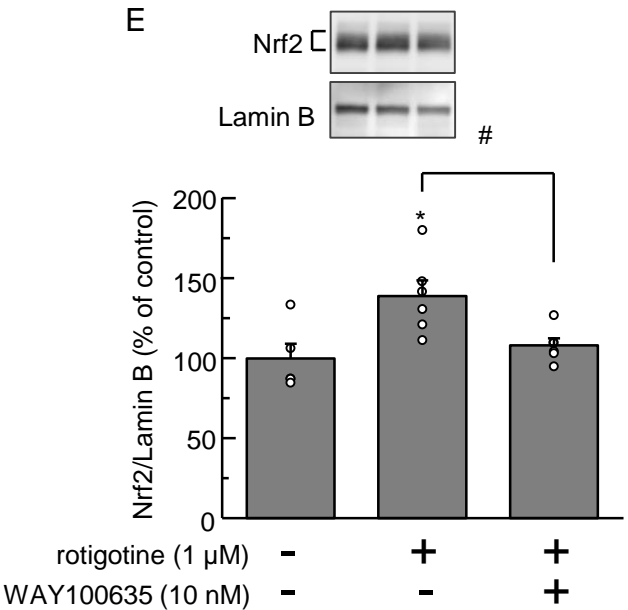
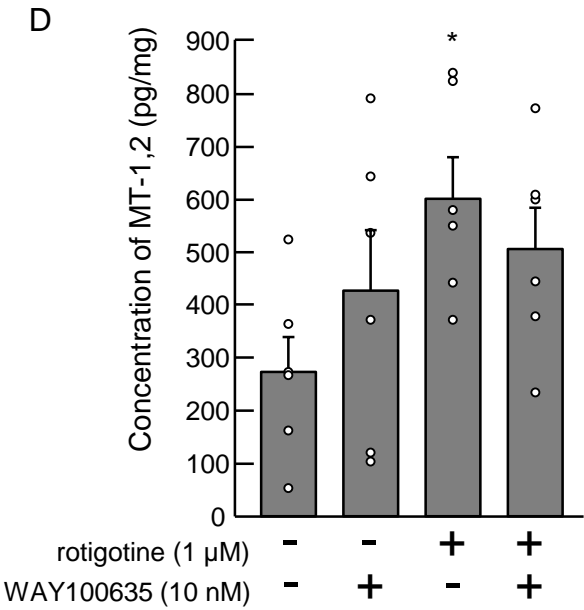
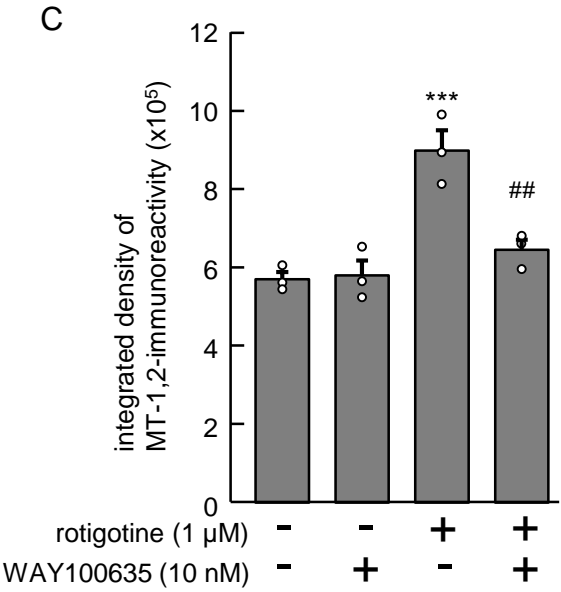
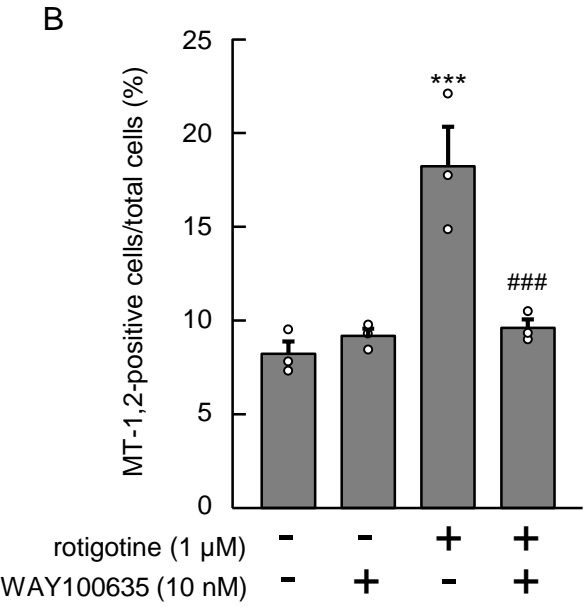
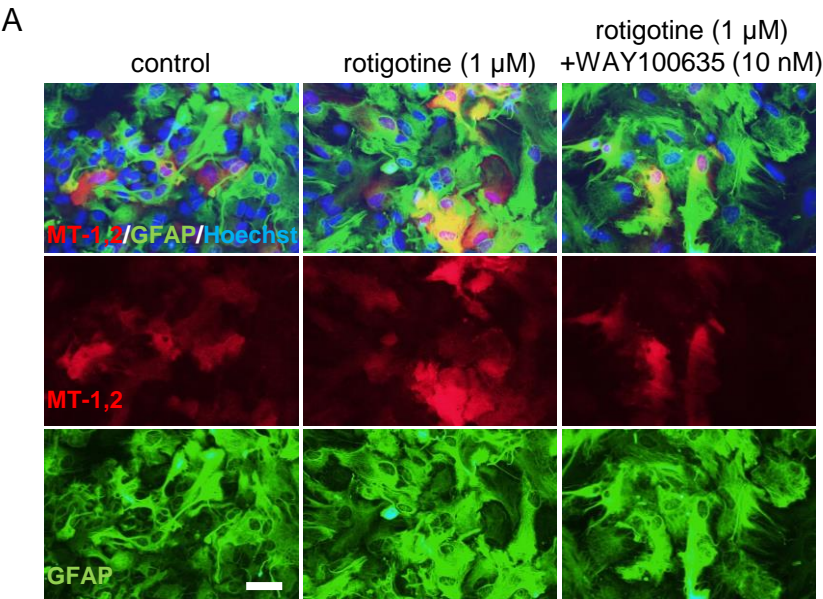


Fig. 3

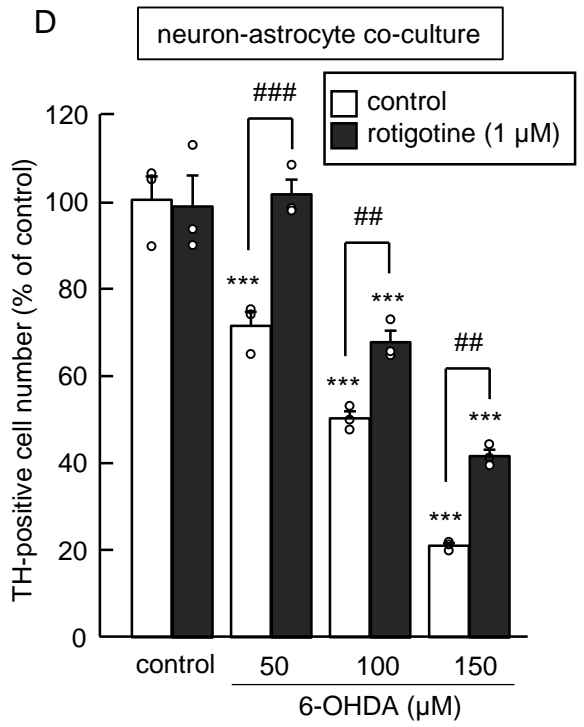
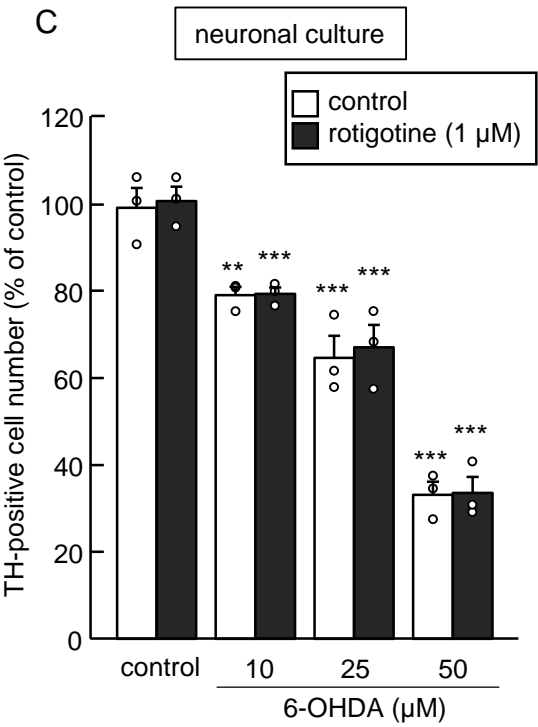
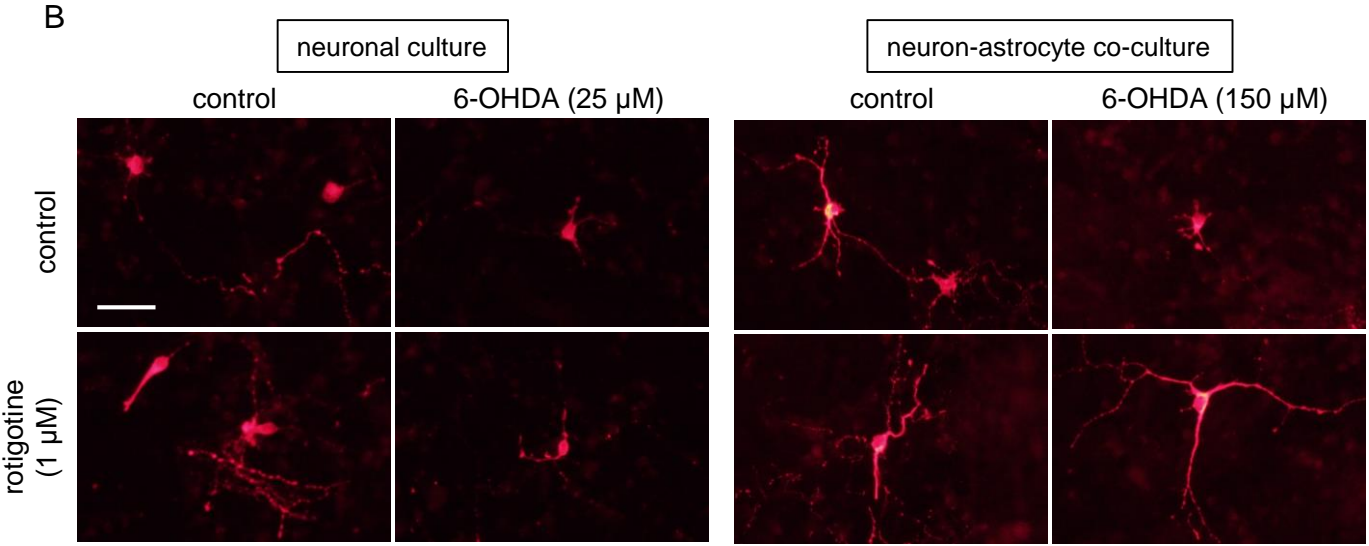
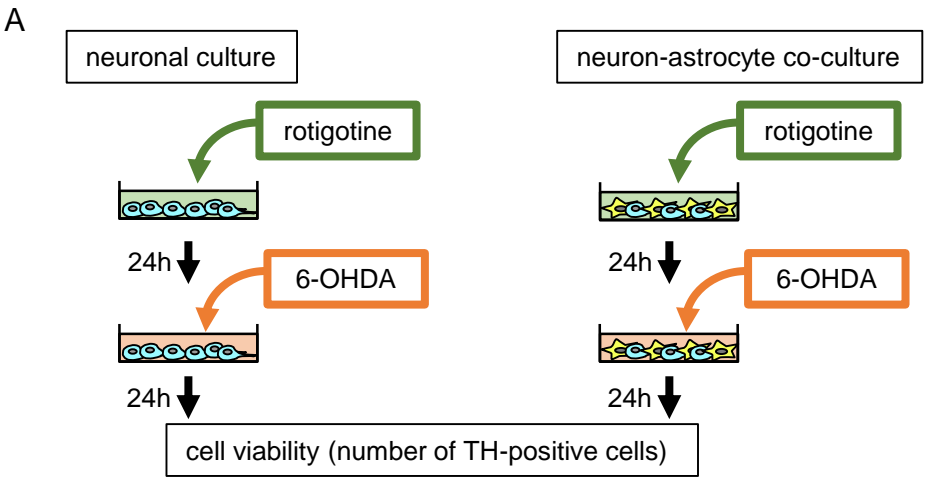
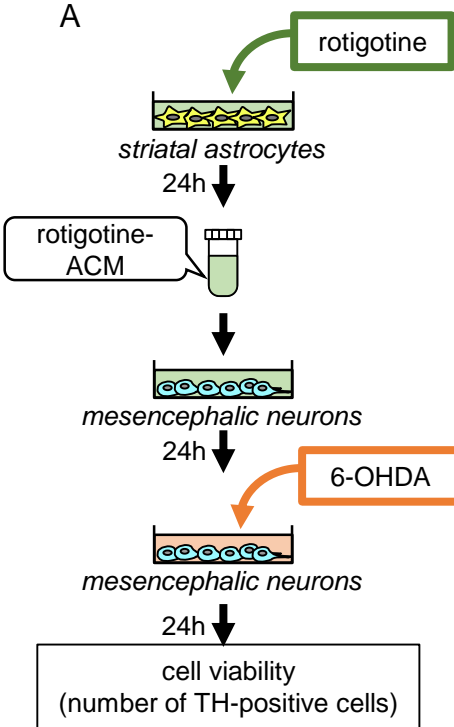
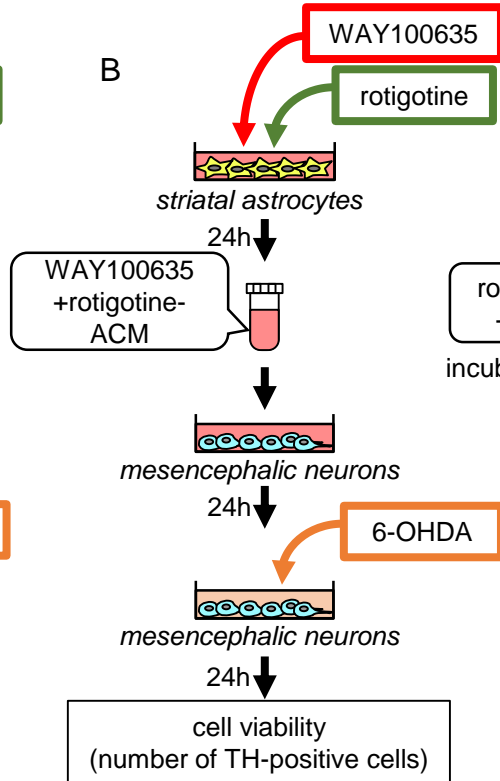


Fig. 4

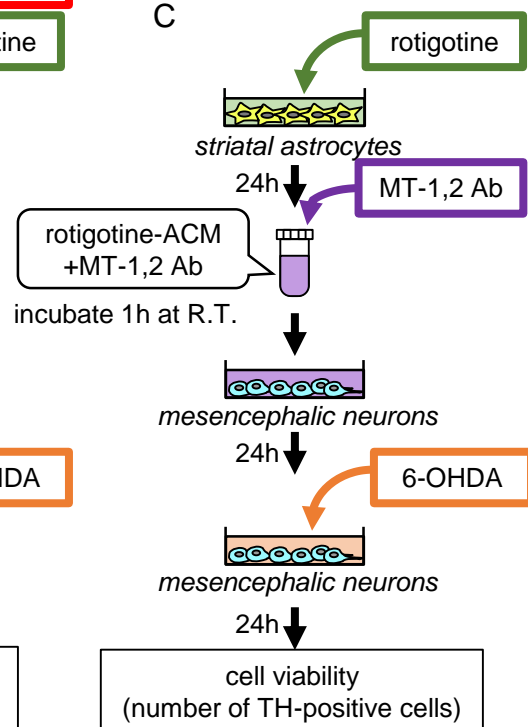
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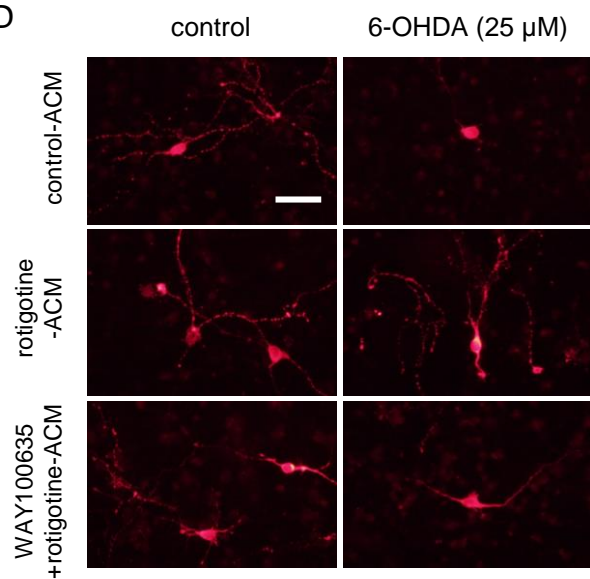
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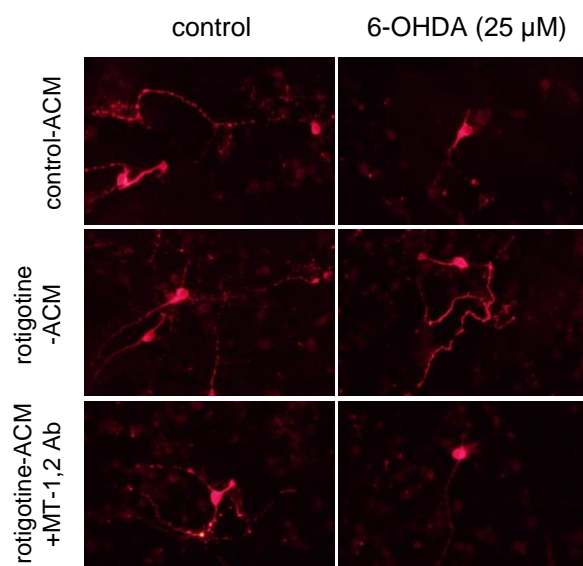
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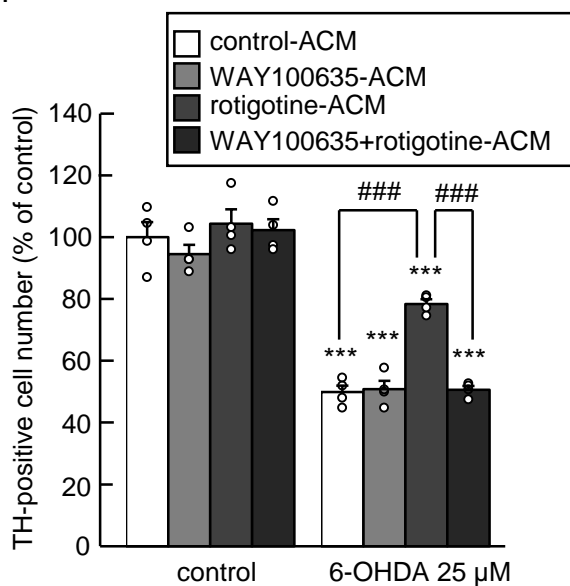
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E



F



G

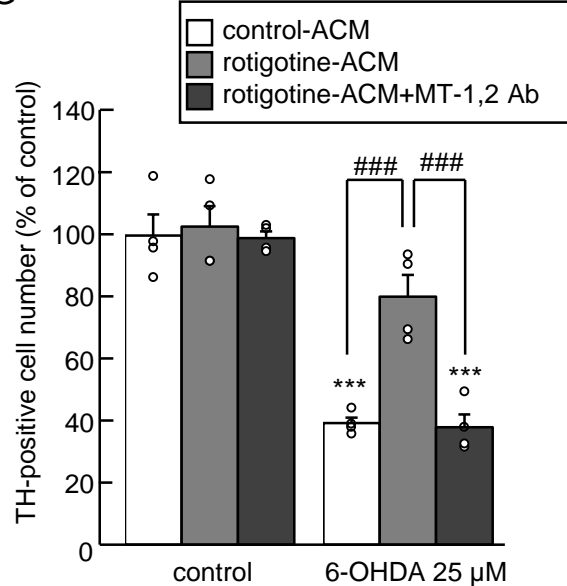


Fig. 5

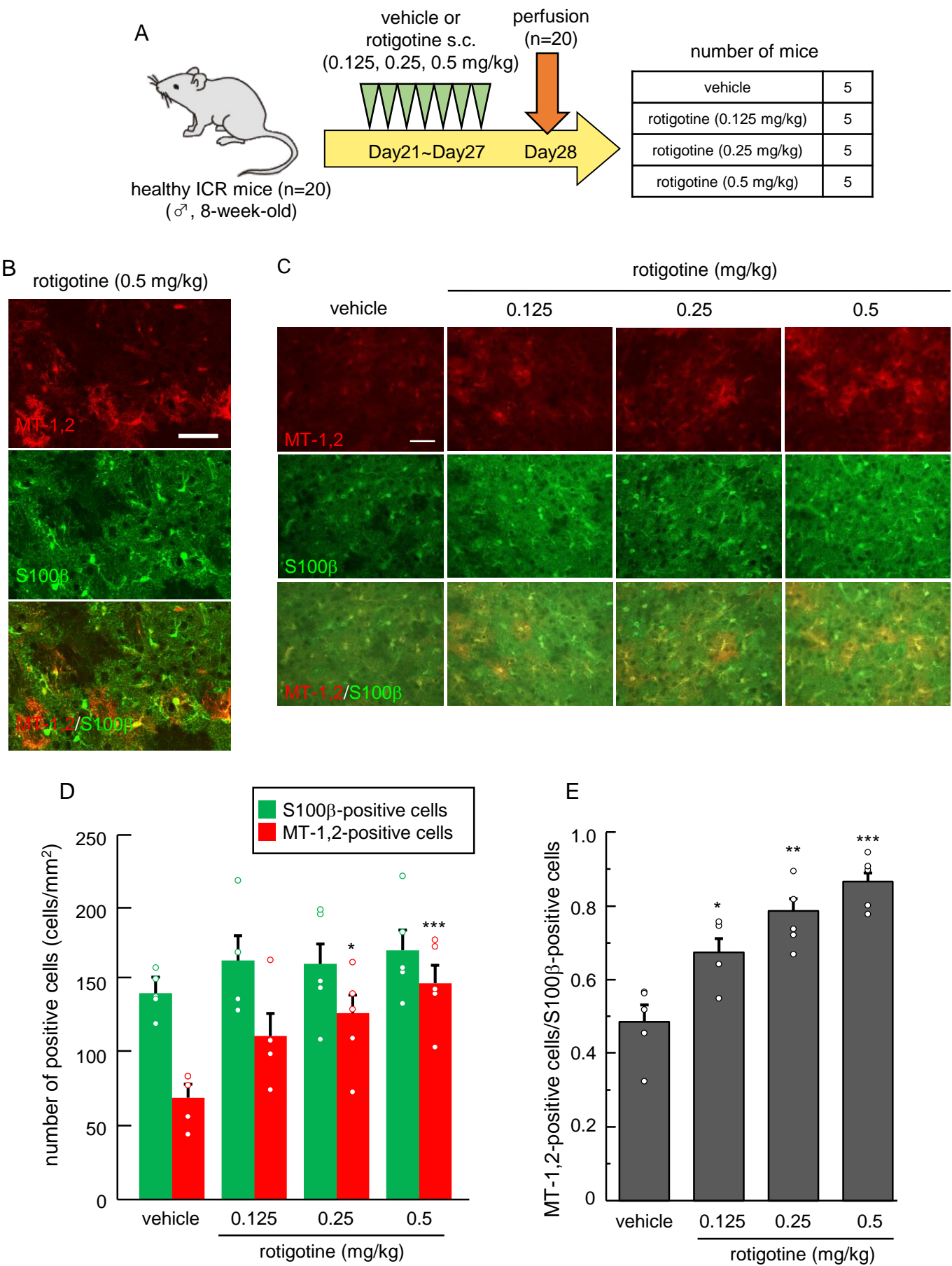


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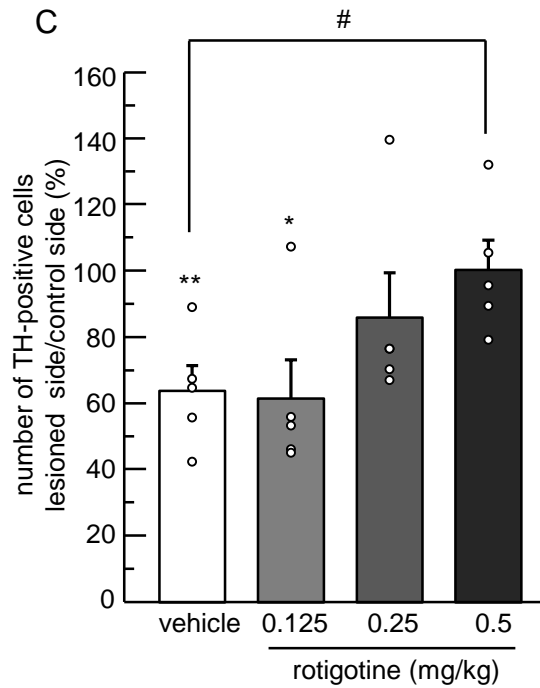
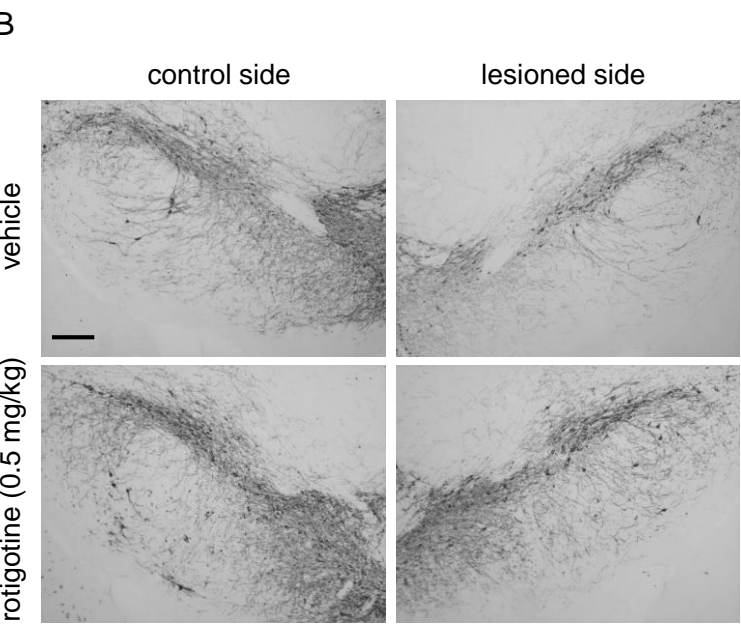
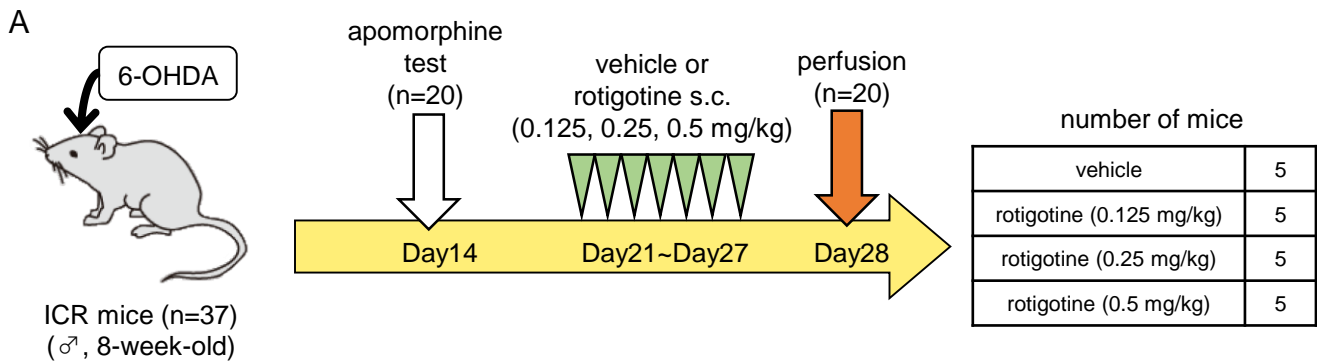


Fig. 7

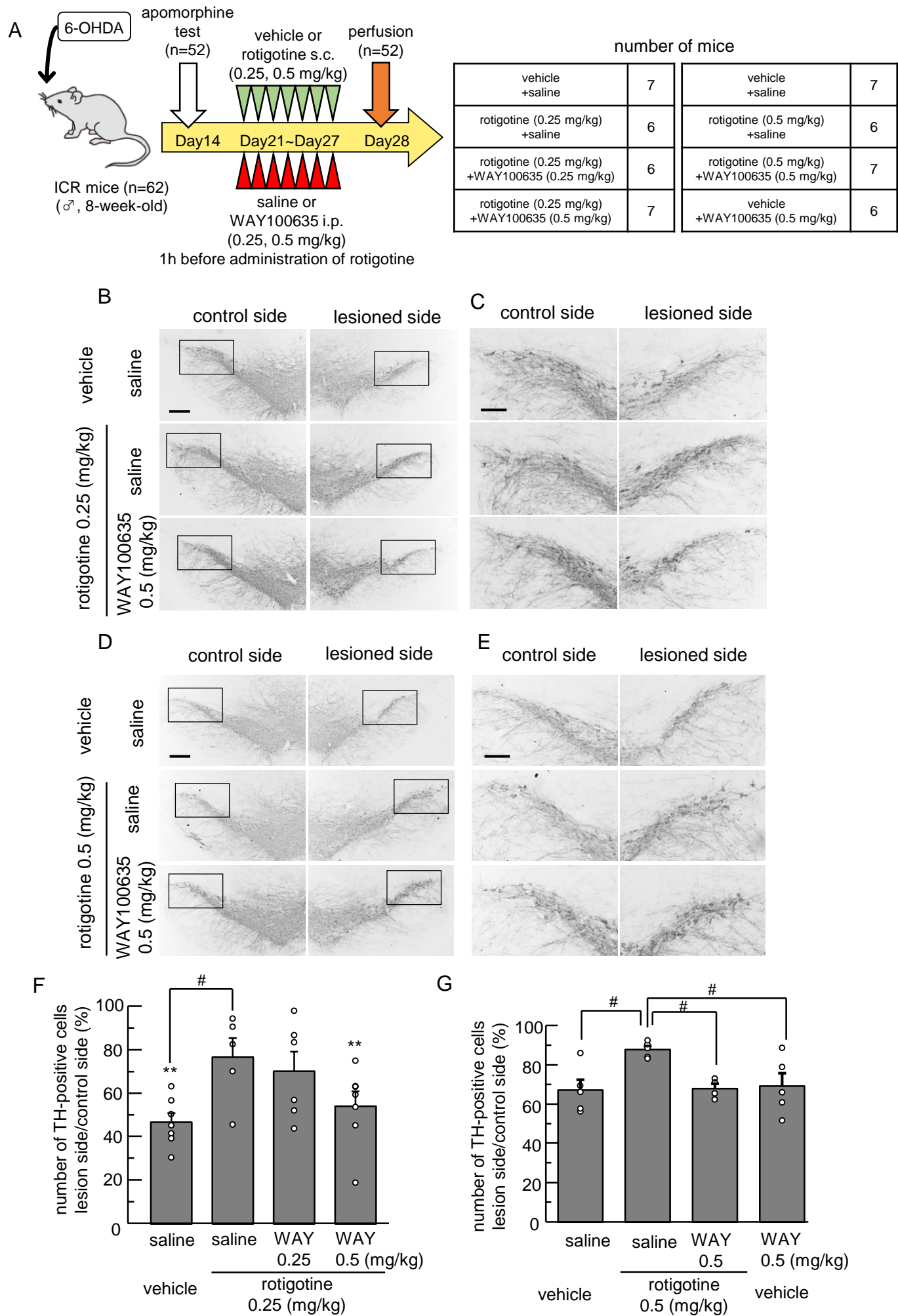
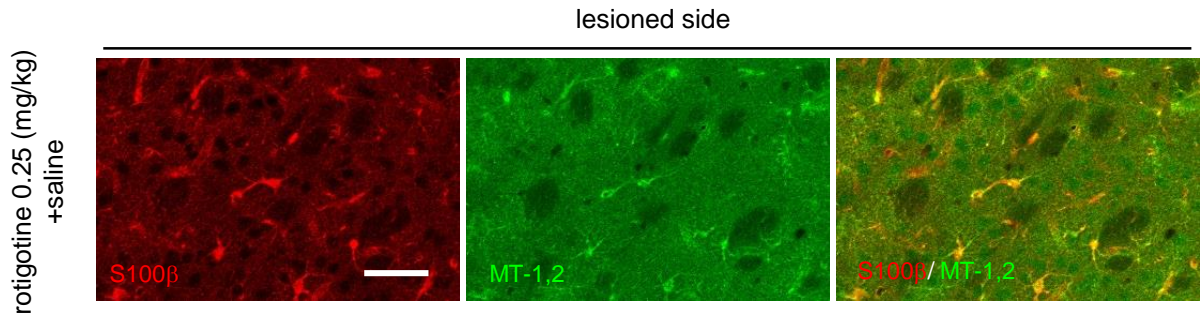
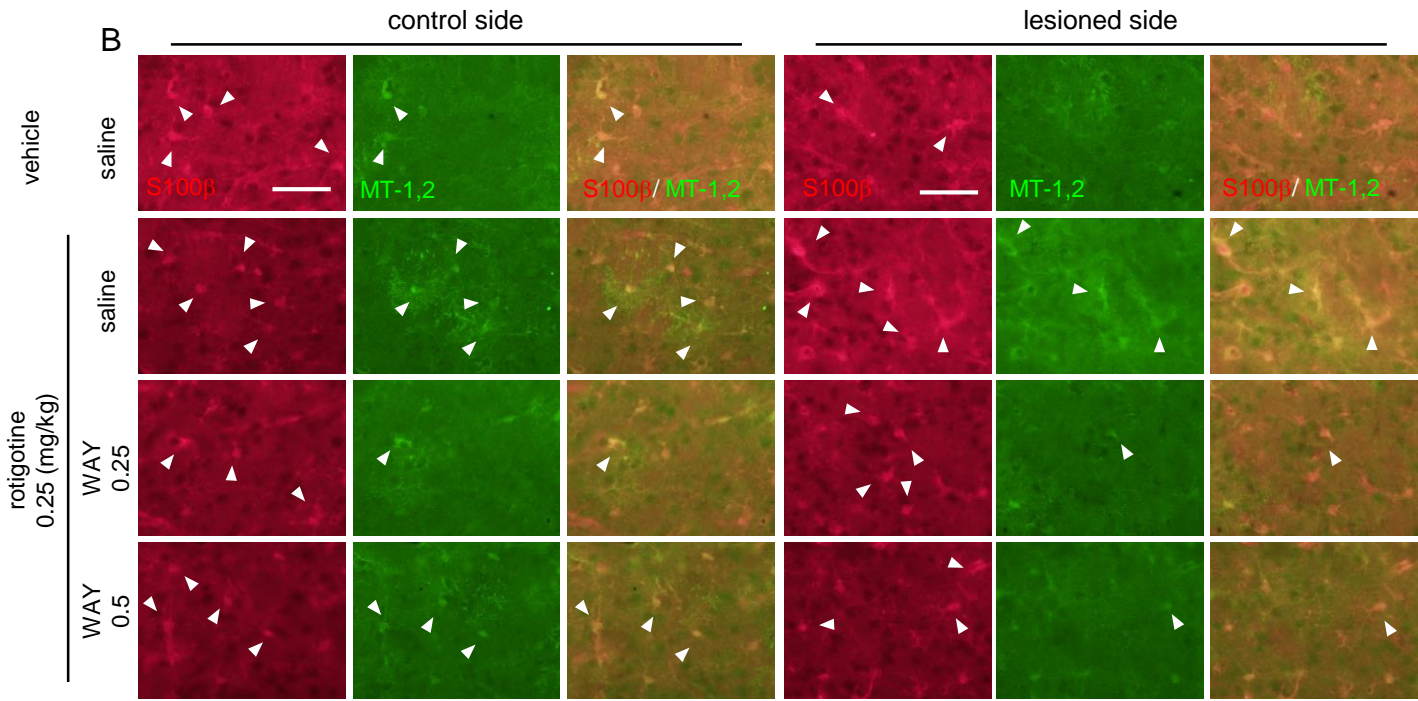


Fig. 8

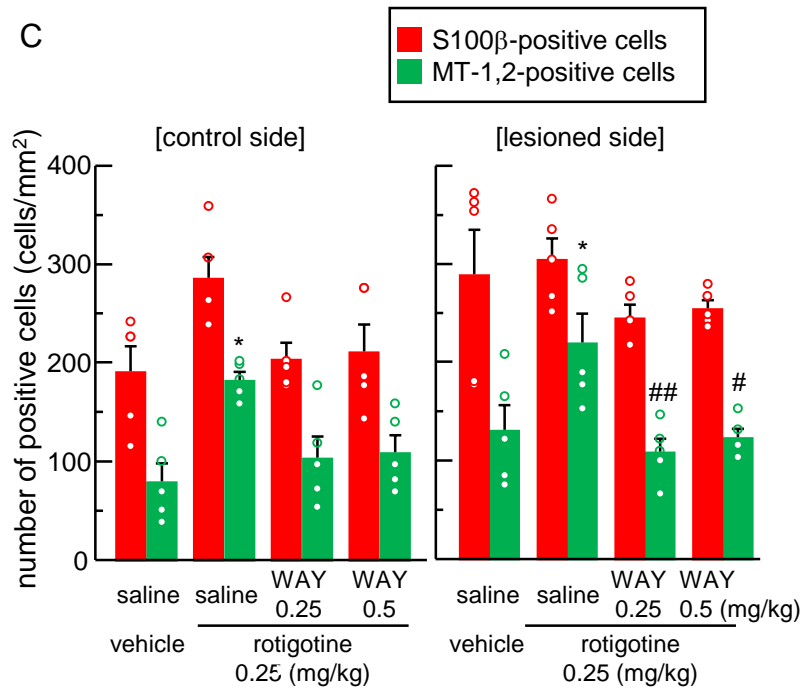
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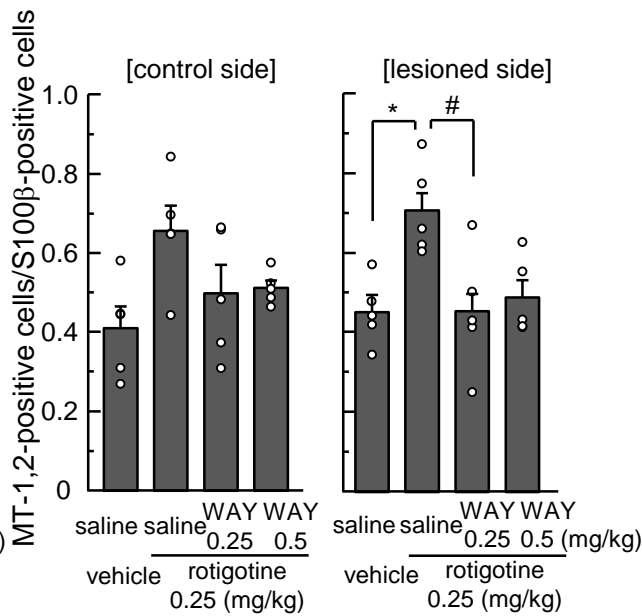
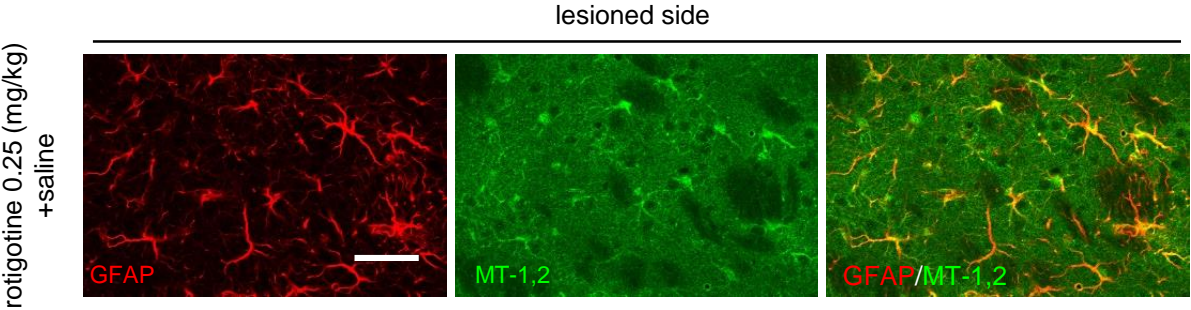
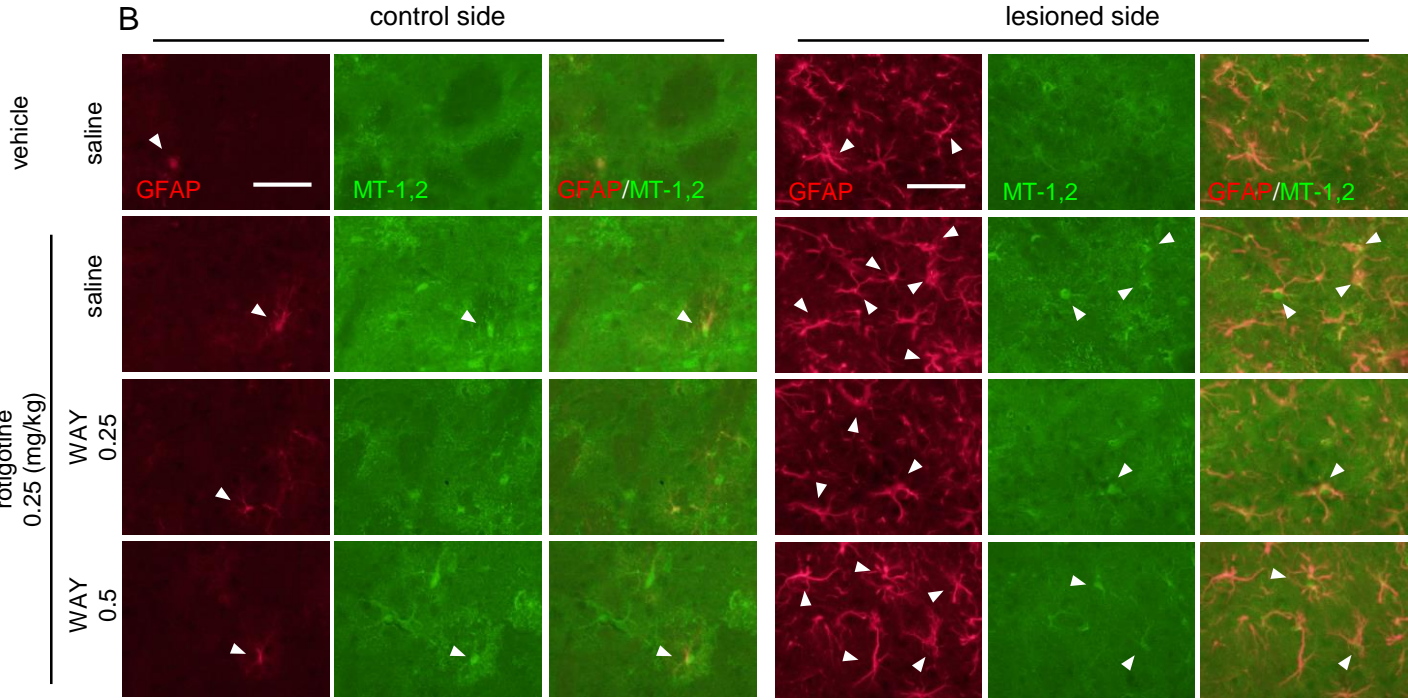


Fig. 9

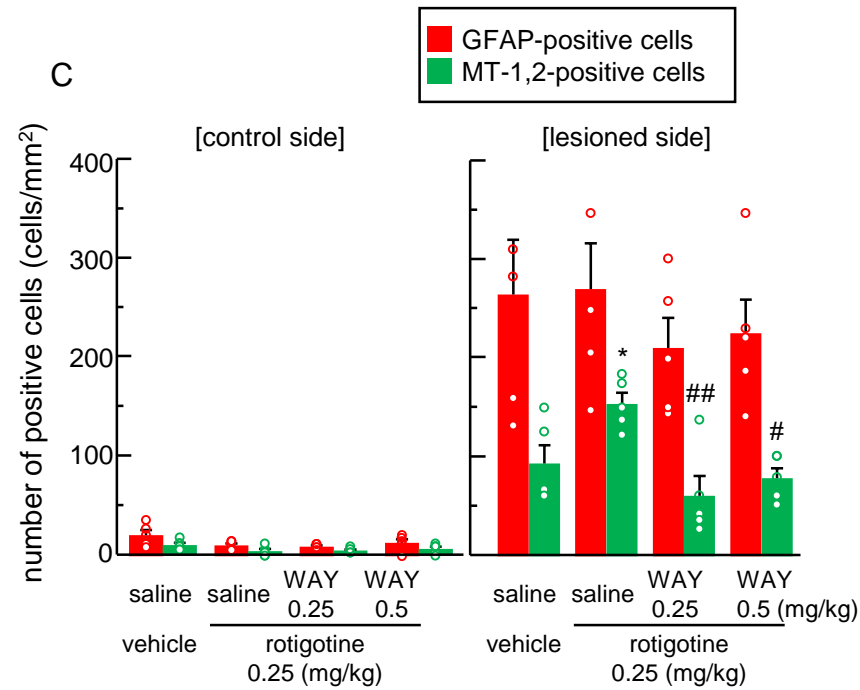
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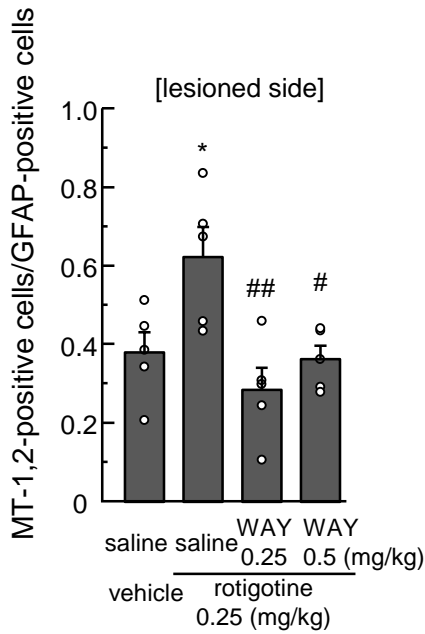
B



C



D



Dopaminergic neuroprotective effects of rotigotine via 5-HT_{1A} receptors: possibly involvement of metallothionein expression in astrocytes

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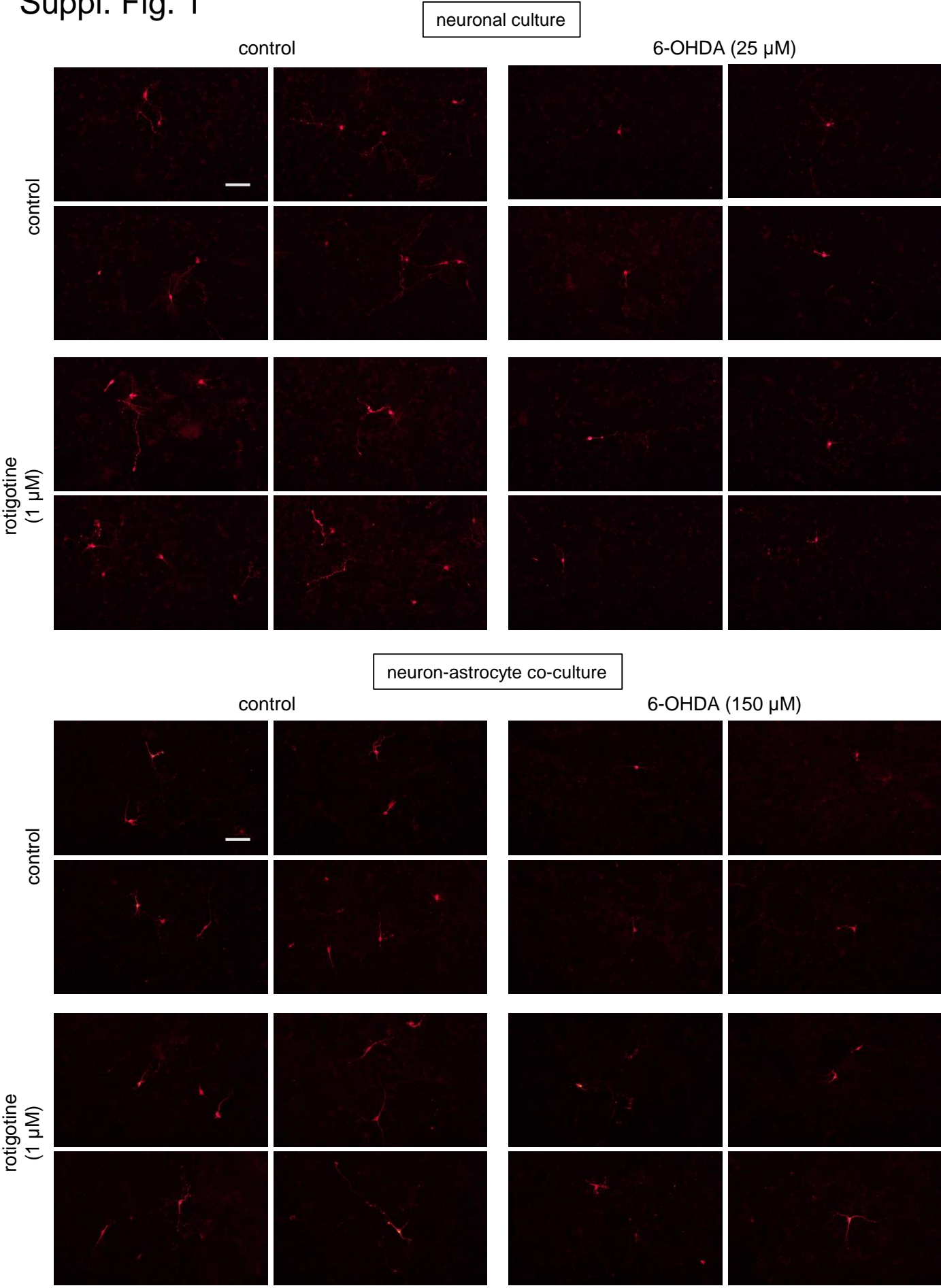
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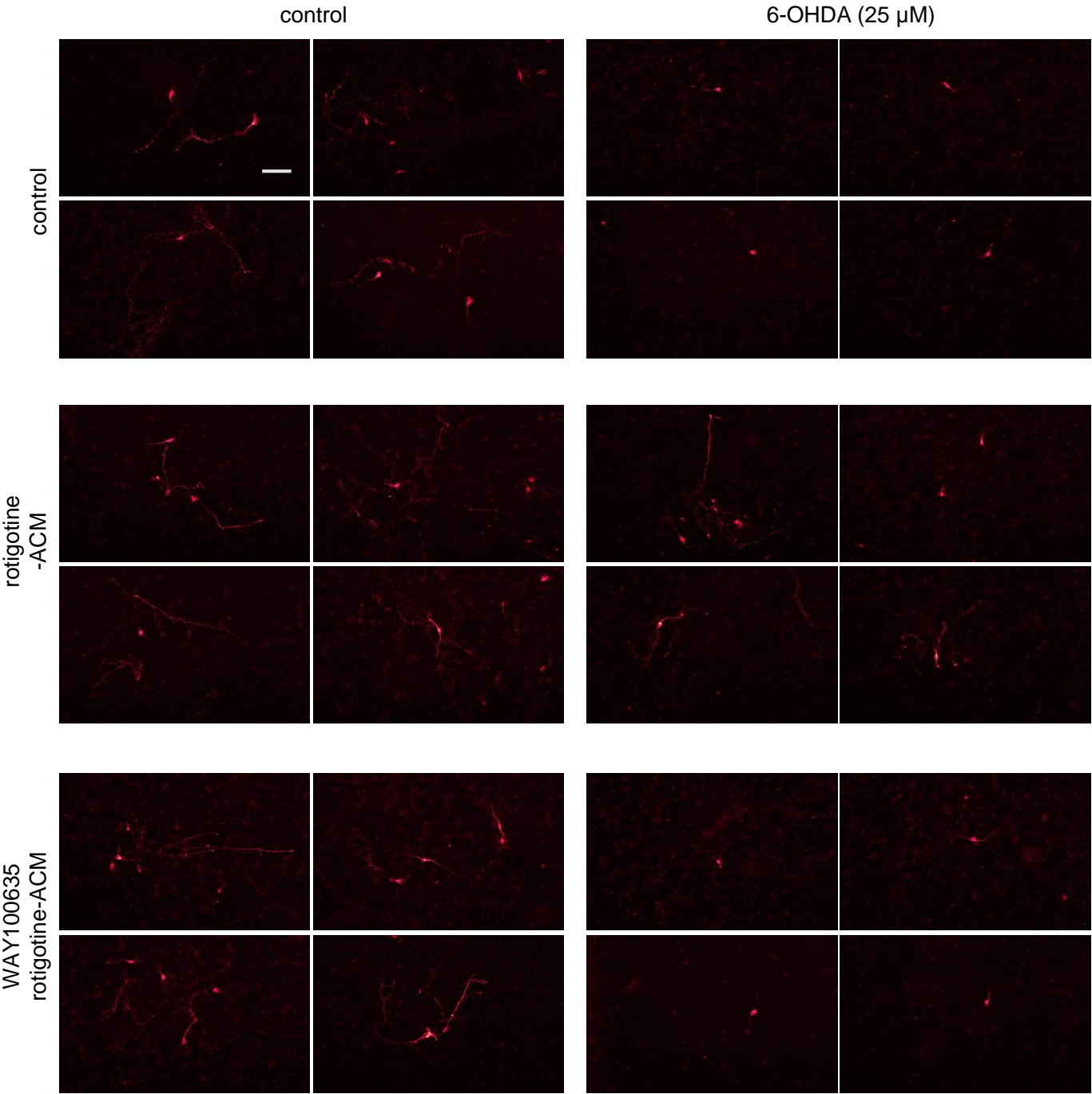
Suppl. Fig. 1



Suppl. Fig. 1 Representative photomicrographs of TH immunostaining. Mesencephalic neuronal culture or mesencephalic neuron and striatal astrocyte coculture were treated with rotigotine (1 μ M) for 24 h, and then exposed to 6-OHDA for 24 h. Scale bar: 100 μ m. Corresponding to Fig. 3B.

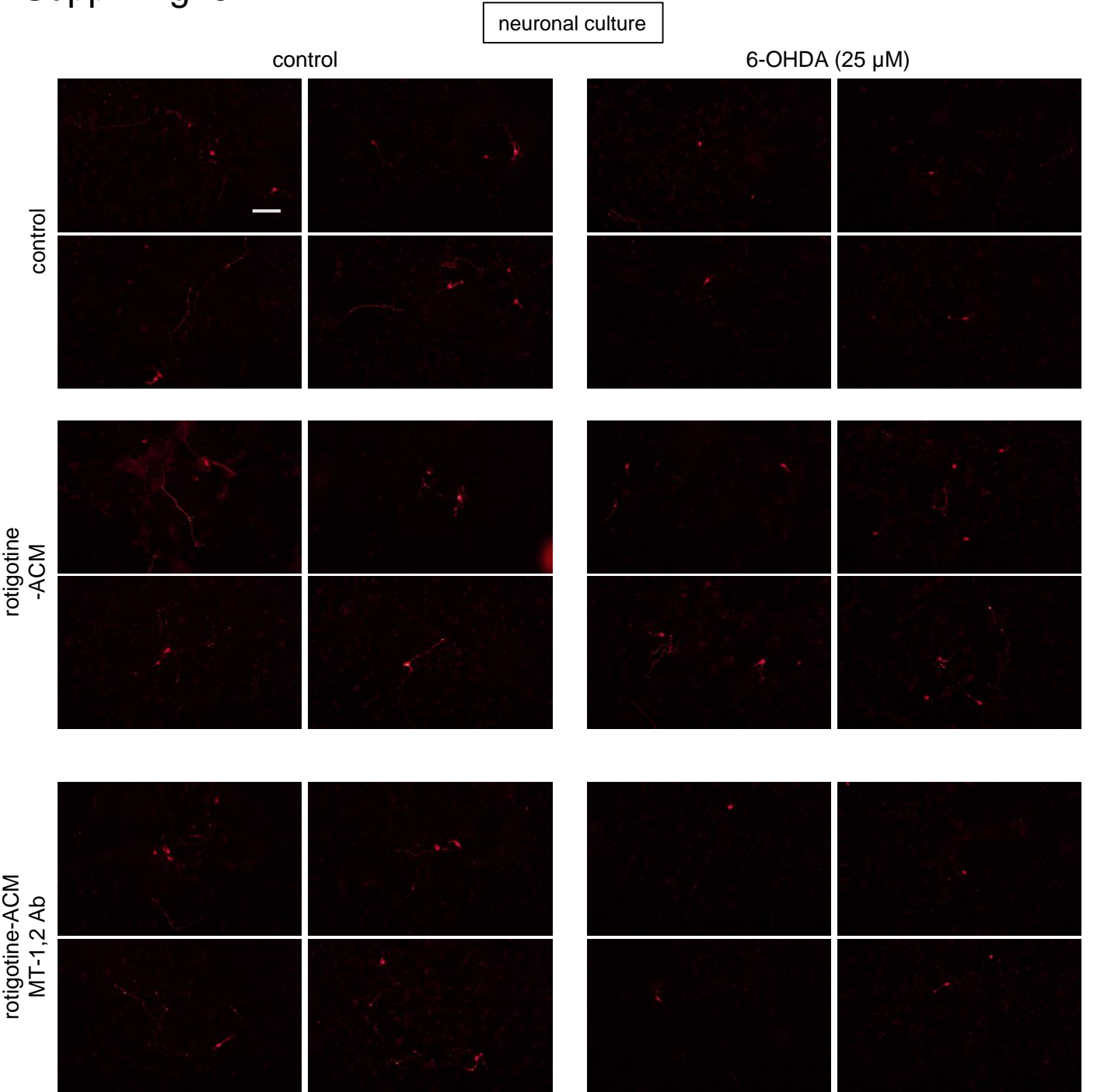
Suppl. Fig. 2

neuronal culture



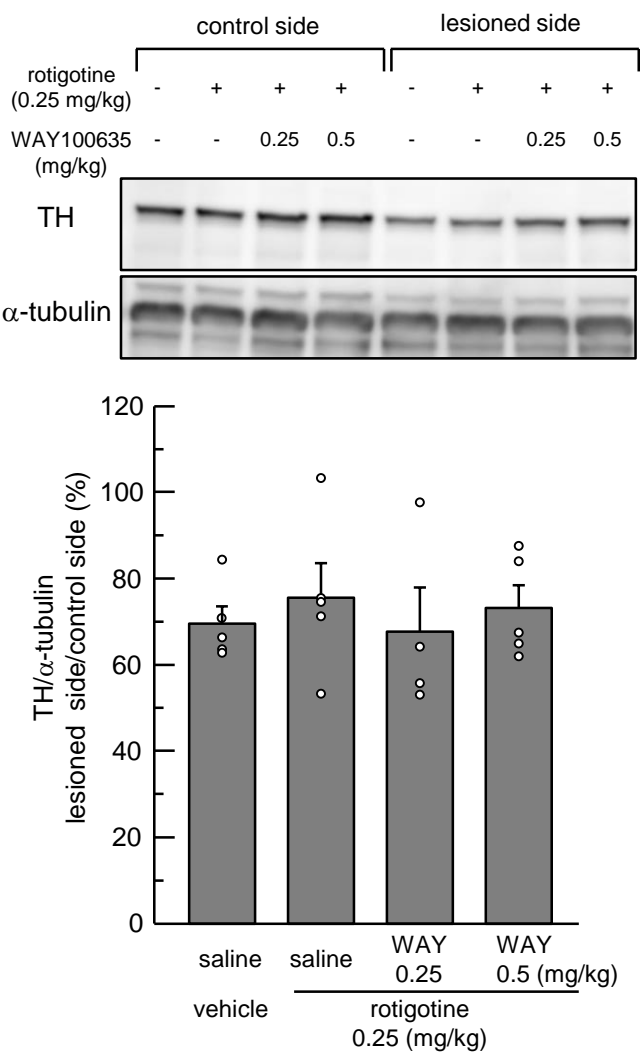
Suppl. Fig. 2 Representative photomicrographs of TH immunostaining. Rotigotine and/or WAY100635 were added to astrocytes cultures and incubated for 24 h. Astrocyte conditioned media (ACM) were collected, and mesencephalic neurons were pre-treated with ACM (control-ACM, rotigotine-ACM, or WAY100635+rotigotine-ACM) for 24 h prior to 6-OHDA exposure. Scale bar: 100 μm. Corresponding to Fig. 4D.

Suppl. Fig. 3



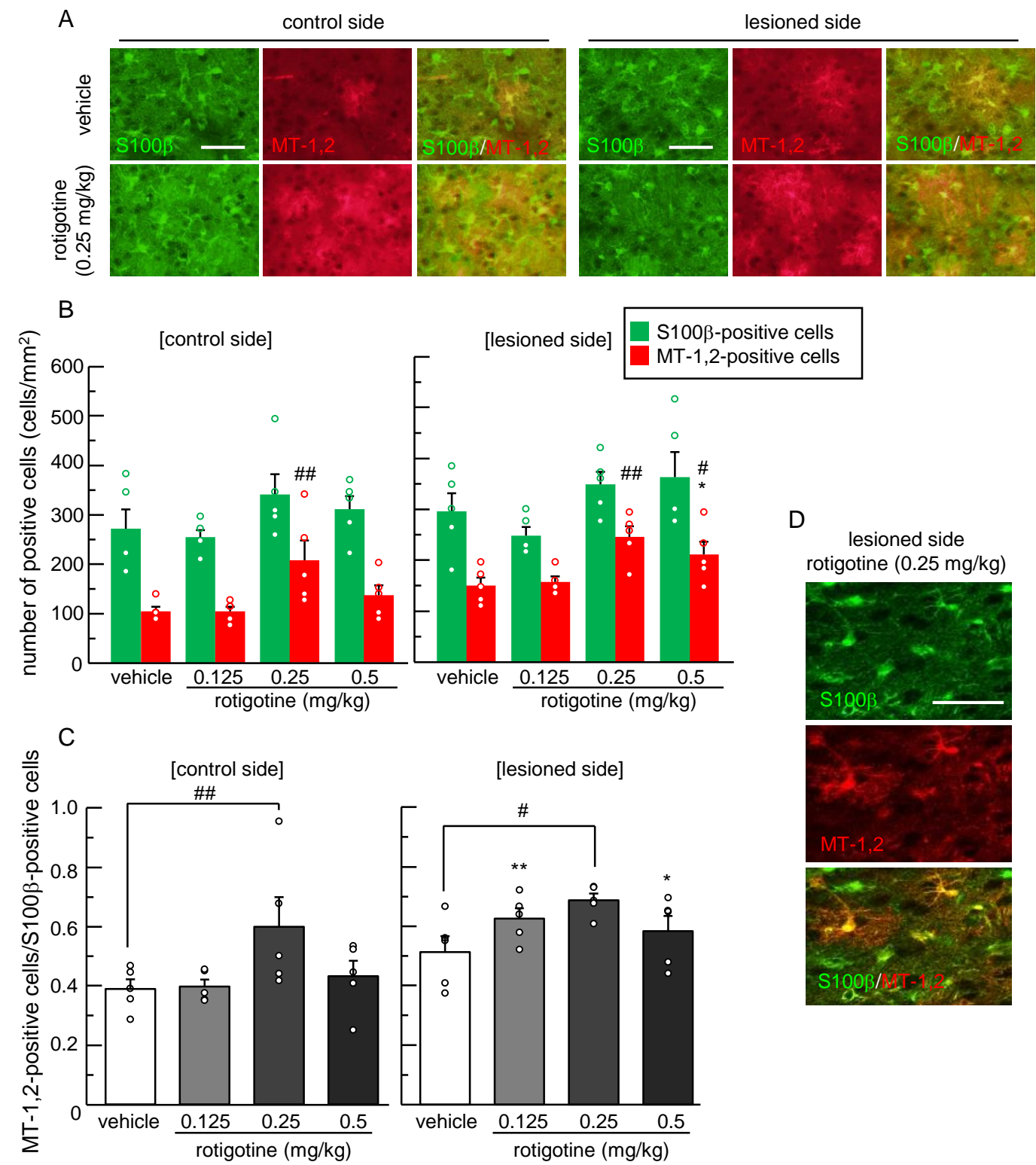
Suppl. Fig. 3 Representative photomicrographs of TH immunostaining. Rotigotine was added to astrocytes cultures and incubated for 24 h. Astrocyte conditioned media (ACM) were collected, and anti-MT-1,2 antibody was added to rotigotine-ACM to neutralize secreted MT-1,2 from rotigotine-treated astrocytes. Mesencephalic neurons were treated with rotigotine-ACM containing anti-MT-1,2 antibody for 24 h, and then exposed to 6-OHDA. Scale bar: 100 μ m. Corresponding to Fig. 4E.

Suppl. Fig. 4



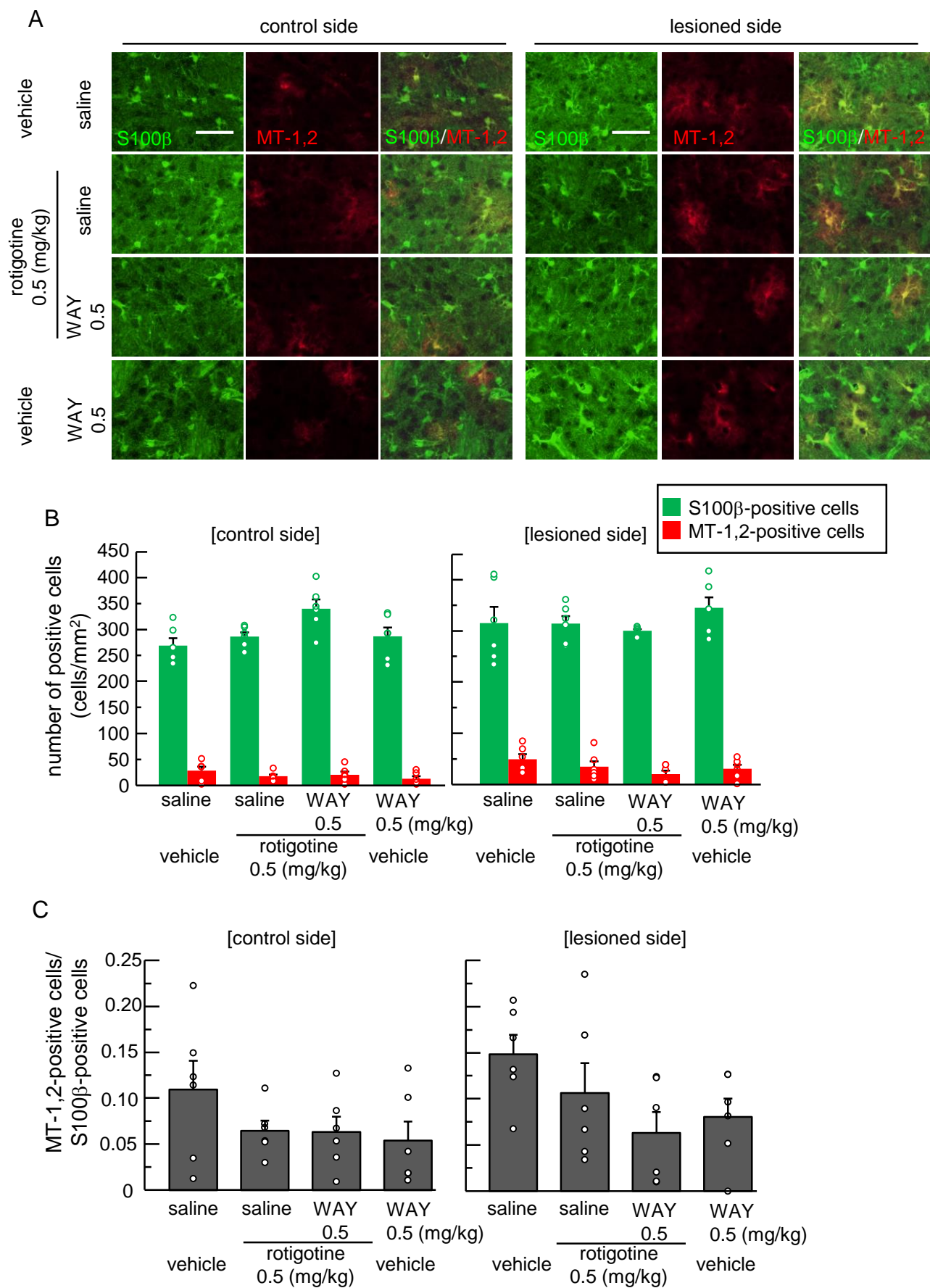
Suppl. Fig. 4 Effects of administration of rotigotine (0.25 mg/kg, s.c.) and WAY100635 (0.25, 0.5 mg/kg, i.p.) for 7days on TH expression in the ventral midbrain of parkinsonian mice. Data are presented as mean \pm SEM (n = 5).

Suppl. Fig. 5



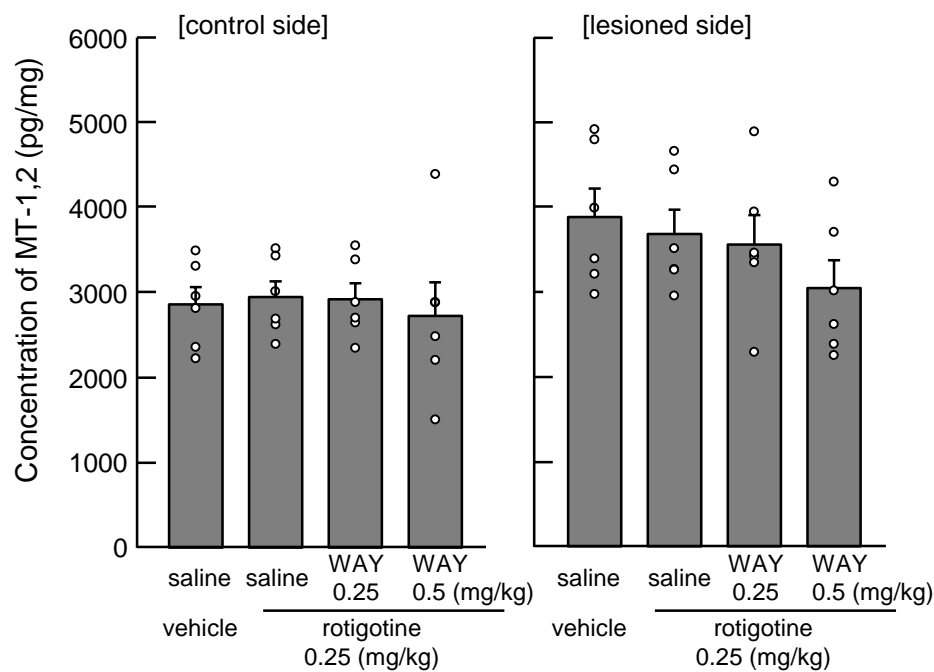
Suppl. Fig. 5 Effects of rotigotine (0.125, 0.25, 0.5 mg/kg/day, s.c.) administrations for 7 days on MT-1,2 expression in the striatal astrocytes in parkinsonian mice. (A) Representative photomicrographs of S100 β and MT-1,2 double immunostaining. Scale bar: 50 μ m. (B) Number of S100 β - and MT-1,2-positive cells in the striatum. (C) The ratio of MT-1,2 positive cells to total S100 β -positive cells. (D) Images of confocal laser microscope. Scale bar: 100 μ m. Data are presented as mean \pm SEM (n = 5) *p < 0.05, **p < 0.01 vs. control side of each group, #p < 0.05, ##p < 0.01 vs. the same side of the vehicle-treated group.

Suppl. Fig. 6

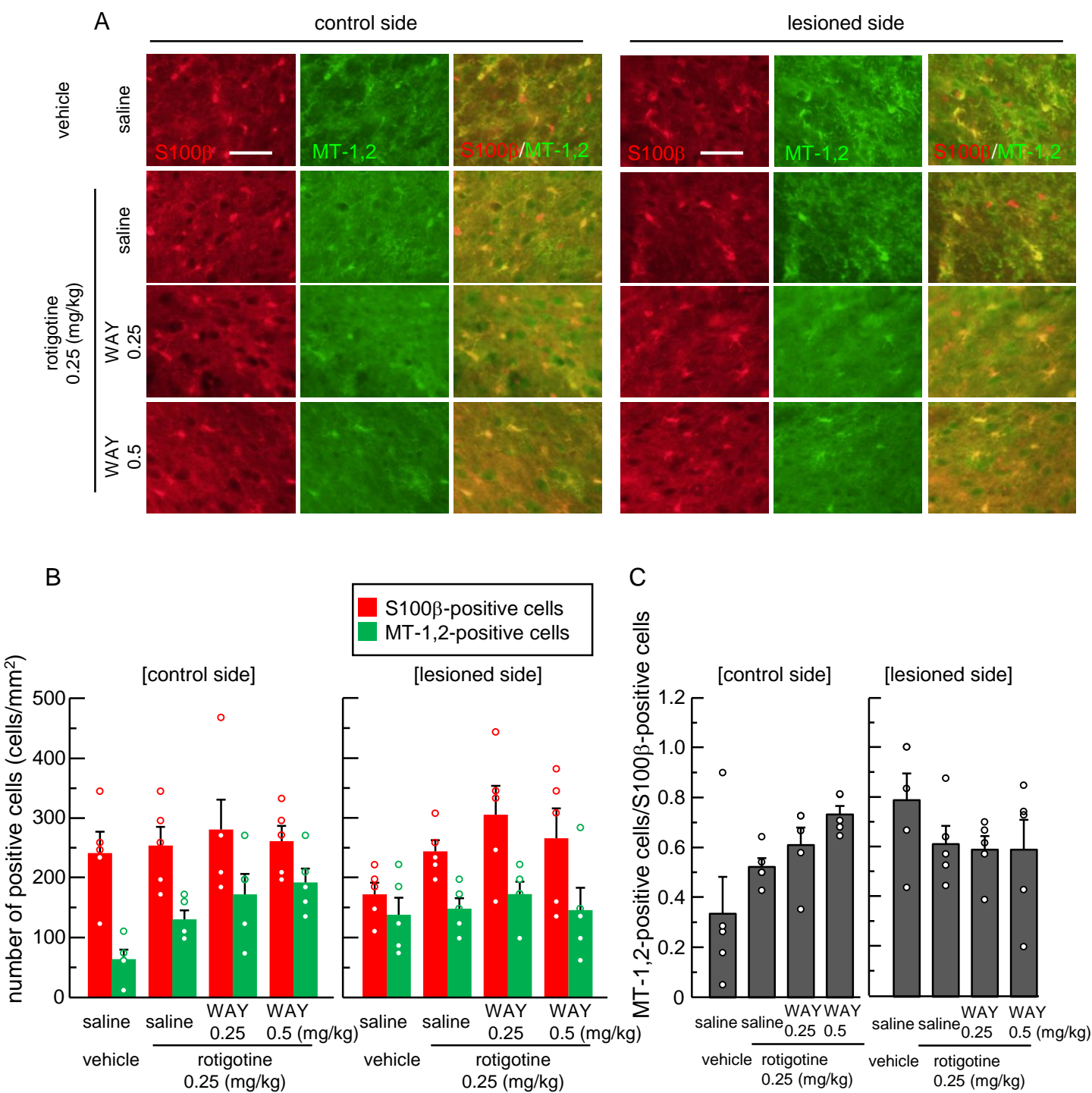


Suppl. Fig. 6 Effects of administration of rotigotine (0.5 mg/kg/day, s.c.) and WAY100635 (0.5 mg/kg/day, i.p.) for 7 days on MT-1,2 expression in the striatum of parkinsonian mice. (A) Representative photomicrographs of S100 β and MT-1,2 double immunostaining. Scale bar: 50 μ m. (B) Number of S100 β - and MT-1,2-immunopositive cells in the striatum. (C) Ratio of MT-1,2-positive astrocytes to total astrocytes. Data are presented as mean \pm SEM (n = 6)

Suppl. Fig. 7



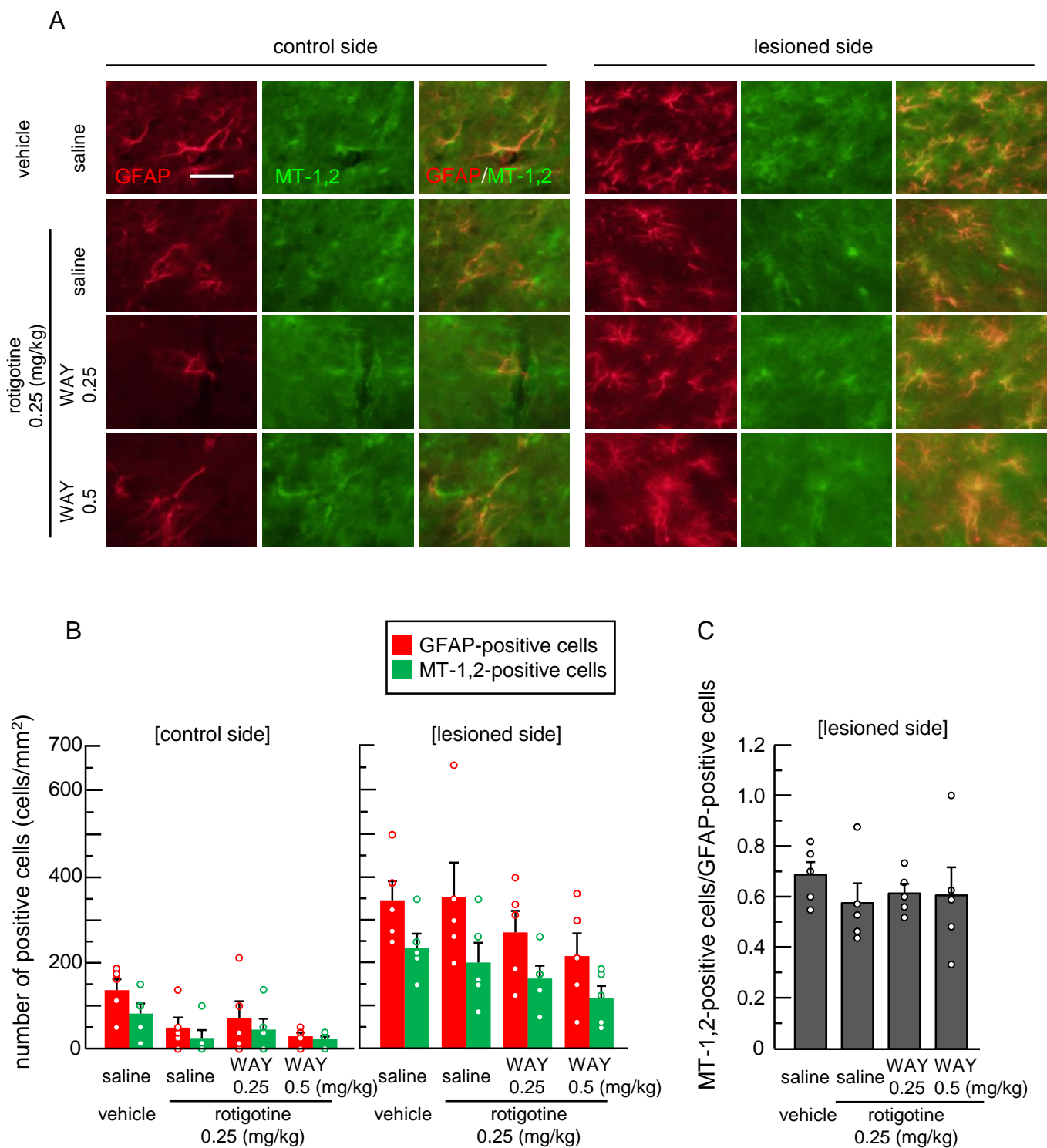
Suppl. Fig. 7 Changes in MT-1 content in total cell lysate from striatum of parkinsonian mice after treatment rotigotine (0.25 mg/kg, s.c.) and/or WAY100635 (0.25, 0.5 mg/kg, i.p.).
Data are presented as mean \pm SEM (n = 6).



Suppl. Fig. 8 Effects of administration of rotigotine (0.25 mg/kg/day, s.c.) and WAY100635 (0.25, 0.5 mg/kg/day, i.p.) for 7 days on MT-1,2 expression in S100 β -positive astrocytes in the SNpc of parkinsonian mice.

(A) Representative photomicrographs of S100 β and MT-1,2 double immunostaining. Scale bar: 100 μ m.

(B) Number of S100 β - and MT-1,2-immunopositive cells in the striatum. (C) Ratio of MT-1,2-positive astrocytes to total astrocytes. Data are presented as mean \pm SEM (n = 5)



Suppl. Fig.9 Effects of administration of rotigotine (0.25 mg/kg/day, s.c.) and WAY100635 (0.25, 0.5 mg/kg/day, i.p.) for 7 days on MT-1,2 expression in the GFAP-positive astrocytes in SNpc of parkinsonian mice. (A) Representative photomicrographs of GFAP and MT-1,2 double immunostaining. Scale bar: 100 μ m. (B) Number of S100 β - and MT-1,2-immunopositive cells in the striatum. (C) Ratio of MT-1,2-positive astrocytes to total astrocytes. Data are presented as mean \pm SEM (n = 5)